PROGNOSTIC FACTORS IN MALIGNANT PLEURAL MESOTHELIOMA

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Abstract

Background and aims: Diffuse malignant mesothelioma of the pleura, also called malignant pleural mesothelioma (MPM), is a rare cancer arising from mesothelial cells. The current clinical tumor, node, metastasis (TNM) staging is based on computed tomography (CT) findings, which lack accuracy, especially in the evaluation of MPM tumor extent. To date, there are no circulating biomarkers available that can be used for diagnosis or to evaluate prognosis. Despite recent advances in the management of MPM overall survival remains poor, ranging from 9 to 12 months. Current treatment options include chemotherapy, radiotherapy, and surgery either alone or in combination. Patient's performance status, age, sex, and histological subtype are currently the strongest clinical prognostic factors that also guide management. The primary aims of this study were to search for radiological, histopathological, and clinical prognostic factors in MPM focusing on the specific tools available in our research environment. In addition, we aimed to define a novel and practical way to estimate tumor size (TS) from a CT and to assess the prevalence of cancer cachexia in MPM patients. Finally, we also aimed to evaluate the role of circulating activins and follistatins and to confirm previously observed preclinical associations in MPM patients.

Material and methods: Using the Finnish Cancer Registry all MPM patients diagnosed in Finland between 2002 to 2012 were identified. They formed the population group used for studies I, III, and IV. Data was collected from various national registries including hospital medical records. In the first study, we reviewed the CT characteristics of 161 patients at the Helsinki University Hospital region. Tumor size was estimated by using the maximal tumor thickness and grading tumor extension along the chest wall. In studies III and IV, we identified a subcohort of patients (n = 52) from the cancer registry with survival over five years, characterized as a long-term survivals (LTS). The epithelioid subgroup from study I formed the control group (n = 84) in these studies. We conducted a thorough clinical and histopathological evaluation in these patients, in order to confirm the initial diagnosis and to search for prognostic features. In study II, we prospectively enrolled 106 patients with suspected thoracic malignancy from June 2016 to January 2018. Pretreatment blood samples were obtained and activins and follistatins levels were quantified using enzyme-linked immunosorbent assay. Since activins are associated with cancer cachexia, we investigated the prevalence of cachexia in MPM. Patients were defined as cachectic if they had prior weight loss with or without CT-based sarcopenia. A subset of MPM patients was evaluated for chemotherapy response. The Cox's proportional hazard model was used to estimate the strength of the association between various factors and survival. Multivariate logistic or linear regression analysis was used to determine the independent influence of circulating biomarkers on cachexia or chemotherapy response. Analyses were performed using the SAS System for Windows (SAS institute, Inc. Cary, NC, version 9.4) or SPSS (IBM SPSS Statistics, Chicago, version 24.0 or 25.0).

Results: We found that TS estimation was reproducible and was associated with sarcomatoid histology and TNM stage. In multivariate analyses, high TS ($p < 0.001$) as well the amount of pleural effusion ($p < 0.001$) were the only CT characteristics associated with survival. Similarly, patients with epithelioid histology had the best prognosis ($p < 0.001$). Within the epithelioid subgroup, we found that TS ($p = 0.004$), performance status ($p < 0.001$) and treatment were associated with survival ($p = 0.005$). Low nuclear grade ($p < 0.001$) and the presence of exophytic polypoid growth ($p = 0.008$) were the only independent histopathological features related to prolonged survival. Twelve (57%) MPM patients were

assessed as cachectic at diagnosis. Among the studied biomarkers, activin A was the best one at separating the main study groups: it was elevated in MPM compared to non-small cell lung cancer or benign lung tumor patients ($p < 0.001$). Activin A associated also with cancer cachexia ($p = 0.001$), pretreatment TS ($r = 0.549$; $p = 0.010$) and chemotherapy response ($p = 0.0028$).

Conclusions: We found that TS, performance status, and histological subgroups were the strongest predictors of mortality. In the epithelioid subgroup, nuclear grade and polypoid growth pattern were the most robust prognostic factors. Finally, we found that activin A could be a useful biomarker in MPM for the association with cancer cachexia, TS, and chemotherapy response.

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List of original publications

This thesis is based on the following publications, which will be referred to in the text by respective Roman numerals:

I Computed tomography in the evaluation of malignant pleural mesothelioma – Association of tumor size to a sarcomatoid histology, a more advanced TNM stage and poor survival
Paajanen J, Laaksonen S, Ilonen I, Wolff H, Husgafvel-Pursiainen K, Kuosma E, Ollila H, Myllärniemi M, Vehmas T
Lung Cancer 2018; 116:73-79

II Elevated circulating Activin A levels in malignant pleural mesothelioma patients are related to cancer cachexia and reduced response to platinum-based chemotherapy
Paajanen J, Ilonen I, Lauri H, Järvinen T, Sutinen E, Ollila H, Rouvinen E, Lemström K, Räsänen J, Ritvos O, Koli K, Myllärniemi M
Clinical Lung Cancer 2020; 21: e142-e150

III Histopathological features of epithelial malignant pleural mesotheliomas in patients with extended survival
Paajanen J, Laaksonen S, Ilonen I, Vehmas T, Mäyränpää M, Sutinen E, Kettunen E, Salo J, Räsänen J, Myllärniemi M, Wolff H
Human Pathology 2020; 98:110-119

IV Clinical features in malignant pleural mesothelioma patients with 5-year survival and evaluation of original diagnoses
Paajanen J*, **Laaksonen S***, Ilonen I, Vehmas T, Mäyränpää M, Sutinen E, Kettunen E, Salo J, Räsänen J, Wolff H, Myllärniemi M
Clinical Lung Cancer (In press)
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Abbreviations

ACTR	activin type receptor
AI	artificial intelligence
AJCC	American committee on cancer
ARDS	acute respiratory syndrome
ASCO	American society of clinical oncology
BAP1	BRCA1 associated protein 1
BMI	body mass index
BTS	British thoracic society
CCI	Charlson comorbidity index
CI	confidence interval
CKDN2A	cyclin-dependent kinase inhibitor 2A
CRP	c-reactive protein
CT	computerized tomography
DFS	disease-free survival
DNA	deoxyribonucleic acid
ECOG	eastern cooperative oncology group
ELISA	enzyme-linked immunosorbent assay
EPP	extrapleural pneumonectomy
ERS	European respiratory society
ESTS	European society of thoracic surgeons
FCR	Finnish cancer registry
FDG	fluorine-18-deoxyglucose
FISH	fluorescence in situ hybridization
FS	follistatin
FSTL3	follistatin-like 3
GDF	growth differentiation factor
Hb	hemoglobin
HPF	high power field
HR	hazard ratio
HUS	Helsinki university hospital
IARC	international agency for research on cancer
IASLC	international association for the study of lung cancer
ICC	intraclass correlation
IHC	immunohistochemistry
IL	interleukin
IMIG	international mesothelioma interest group
IMRT	intensity-modulated radiation therapy
IQR	interquartile range
LMM	localized malignant mesothelioma
LTS	long-term survival
MM	malignant mesothelioma
miRNA	micro-ribonucleic acid
MPF	megakaryocyte potentiating factor
MPM	malignant pleural mesothelioma
mRECIST	modified response evaluation criteria in solid tumors
MRI	magnetic resonance imaging
NSCLC	non-small-cell lung cancer

OS	overall survival
PFS	progression-free survival
PET	positron emission tomography
P/D	pleurectomy/decortication
PD-L1	programmed death ligand 1
PS	performance status
QoL	quality of life
RNA	ribonucleic acid
RR	response rate
RT	radiotherapy
SD	standard deviation
SEER	surveillance, epidemiology, and end results
SMI	skeletal muscle index
SMRP	soluble mesothelin related peptides
STEM	scanning transmission electron microscopy
SUV	standardized uptake ratio
TGF- β	transforming growth factor-β
TGV	total glycolytic volume
TMA	tissue microarray
TNM	tumor, node, metastasis
TP53	tumor protein 53
TS	tumor size
TV	tumor volume
UICC	union for international cancer control
US	ultrasound
VATS	video-assisted thoracic surgery
VEGF	vascular endothelial growth factor
WDPM	well-differentiated papillary mesothelioma
WHO	world health organization

1. Introduction

Malignant mesothelioma (MM) is a cancer arising from mesothelial cells. Pleura is the most common location, followed by the peritoneum, and in rare cases the pericardium and tunica vaginalis. The incidence of MM in Finland is slowly declining, with approximately 80 to 100 annual new MM diagnosis [1]. Previous asbestos exposure is the most common risk factor of MM, which is believed to cause nearly 80% of cases worldwide.

Malignant pleural mesothelioma (MPM) patients typically present with dyspnea, which is frequently associated with chest pain, cough, signs of systematic disease such as weight loss, anorexia, malaise, and fatigue [2]. The most common clinical finding in MPM patients is ipsilateral pleural effusion, which is the main reason for dyspnea. The mesothelioma spreads mainly through local invasion, and distant metastasis occurs usually only in advanced stages of the disease.

Imaging plays an essential role in the diagnostic assessment, guidance of treatment decisions, and follow-up evaluation of MPM patients. The radiological appearance of MPM is non-specific and can range from pleural effusion to pleural thickening and to a lobulated mass. An irregular rind-like growth pattern makes the radiological assessment challenging. Contrast-enhanced computed tomography (CT) of the chest and upper abdomen is the main imaging modality. In some cases, magnetic resonance imaging (MRI) or positron emission tomography (PET) can provide useful information [3]. The 8th edition of the American Joint Committee on Cancer/Union for International Cancer Control the tumor, node, and metastasis (TNM) staging system is currently recommended for staging the disease [4]. Previous studies have suggested that measurements of tumor thickness and/or tumor volume by CT could not only help assess treatment response but also have prognostic value, and therefore could supplement the current tumor burden evaluation [5,6].

Multiple circulating biomarkers have been studied regarding diagnosis, disease monitoring, prognosis, and screening. Previous studies have identified that serum levels of mesothelin or soluble mesothelin-related peptides, megakaryocyte potentiating factor, osteopontin, and fibulin-3 are elevated in MPM compared to asbestos-exposed or secondary pleural malignancies [7]. However, multiple confounding factors exist, and none of these markers are accurate enough for clinical use. Thus to date, circulating biomarkers do not contribute to the current diagnostic or treatment guidelines [8,9]. Activins belong to the transforming growth factor-ß superfamily and control cell proliferation and differentiation, among other biological functions. Activin signaling has multiple roles in carcinogenesis, and previous preclinical studies have found that both activin A and B are overexpressed in MPM tumor tissue and cell lines [10,11]. Similarly, a clinical study found that circulating activin A was elevated in MPM compared to healthy controls and is associated with poor prognosis in the epithelioid subtype [12]. Furthermore, previous animal and clinical studies have shown that the activin pathway is associated with cancer cachexia, cancer-related bone loss, and resistance to platinum-based chemotherapy [13–15]. These findings suggest that activins may be interesting biomarkers that could aid diagnosis and have possible prognostic or therapeutic value.

Diagnosis can be difficult due to the rarity and heterogeneity of the tumors. The cornerstone of the diagnosis is the histopathological confirmation of invasive tumor growth, together with clinical and radiological findings pointing towards a diagnosis of MPM. The most reliable tumor tissue sample can

be achieved via thoracic surgery, mostly video-assisted thoracoscopic surgery (VATS). Other diagnostic options include transthoracic needle biopsy or medical thoracoscopy. Differential diagnosis consists of other primary pleural tumors, pleural metastasis of other origins, benign reactive mesothelial proliferation, and other mesotheliomas [16]. Immunohistochemical staining is the most important supplementary technique that discriminates MPM from other benign or malignant pleural diseases [17].

MPM is usually diagnosed in its advanced stage, when treatment options are scarce. Even in the early stages of the disease, it is not possible to achieve a microscopically complete (R0) surgical resection due to unique growth patterns in the pleural cavity [18]. Thus, the current treatment options include chemotherapy, radiotherapy, and surgery either alone or in combination [8]. Despite the progress in treatments in recent years, the prognosis of MPM patients remains dismal. The overall survival in population based studies varies from 9 to 12 months, and a 5-year survival rate is approximately 5% [19,20]. The longest survival times have been reported on patients undergoing multimodality treatments, with median survivals ranging typically from 19 to 25 months [21]. Long-term survival (LTS) is rare, and has been studied mainly in patients treated with surgery or multimodality treatment regimen [22]. The proposed prognostic factors include clinical variables, radiological parameters at presentation, and pathological or molecular findings in the tumor tissue. The most consistent clinical characteristics of prolonged survival patients are young age, absence of weight loss, female sex, and good performance status [20,23]. A small radiological tumor burden assessed either by TNM stage, tumor thickness, or volumetry is also associated with a better prognosis [6,24]. Histological subtyping of MPM is one of the most important prognostic factors and the epithelioid subtype is the most common form with the best prognosis [25]. Within the epithelioid subtype, several different prognostic features have been proposed in the literature, such as a nuclear grading scale and morphological subtypes [17].

The primary purpose of this study was to confirm previously observed findings as well as to look for novel, patient-related, radiological, and histopathological prognostic factors in MPM. To do so, we identified and verified a subset of MPM patients from the Finnish Cancer Registry. Data was collected and combined using the social security number from various governmental registers including individual hospital medical records. Secondly, we studied a set of novel biomarkers, namely activins and follistatins, in a prospective cohort that was collected with the newly established Helsinki Biobank. We aimed to confirm previous findings, in their association with MPM, and clinically important endpoints such as stage, tumor size, cancer cachexia, and chemotherapy response.

2. Review of the literature

2.1 Pleural anatomy and physiology

2.1.1 Pleural anatomy

The pleural mesothelium is a monolayer of mesothelial cells derived from the embryonic mesoderm. Mesothelial cells rest on a submesothelial layer of connective tissue with a basal lamina following a well-defined elastic layer. Under it there is loose connective tissue layer containing adipose tissue, mononuclear cells and blood vessels, nerves and lymphatic lacunas. The deepest layer, a fibroelastic layer, merges the pleura with underlying structures [26]. The pleura is divided into the visceral and parietal pleura, which are united in the hilum to form the pleural cavity [27]. The parietal pleura covers the chest wall, diaphragm and mediastinum, whereas the visceral pleura lines the outer surface of the lung including interlobar fissures [28]. The normal combined thickness of the pleura is 0.2 to 0.4 millimeters, while the pleural space width is 10 to 20 micrometers [29].

2.1.2 Physiology of the pleura

A thin layer of liquid is present between the pleural surfaces and acts as a lubricant during respiratory movements. It is estimated that approximately 0.25 ml of pleural fluid per kilogram of body weight is produced daily. The parietal pleura is responsible for the majority of pleural fluid turnover. The amount of pleural cavity fluid is dependent on the balance of hydrostatic and oncotic pressure differences between the systemic and pulmonary circulations and pleural space [30]. The fluid is produced through the parietal pleura by net filtering pressure gradient and reabsorbed by lymphatic vessels [31]. Most pleural diseases manifest by progressive pleural effusion either by incremental production or decreased reabsorption.

Traditionally, the mesothelial layer was seen as a protective layer with no physiological function. However, it is now recognized to be responsible for the majority of pleural functions including fluid and cell transportation, inflammatory response, tissue repair, and antigen presentation [32]. Mesothelial cells are metabolically active cells that produce cytokines and growth factors such as TGF-ß, nitric oxide, glycosaminoglycans, and surfactant [33].

2.2 Malignant pleural mesothelioma (MPM)

2.2.1 Definition

Malignant mesothelioma (MM) is a type of cancer arising from mesothelial cells which line the pleural cavity and also the peritoneum, pericardium and tunica vaginalis. From these sites, pleural mesothelioma is the most common, including approximately 75%-80% of diagnosed MM cases, in contrast to peritoneal which include 10% to 20% and single reported cases of pericardial and tunica vaginalis [34]. The epidemiology, pathogenesis, prognosis, and clinical behavior differs depending on the origin of the disease.

The term malignant pleural mesothelioma (MPM) refers to a diffuse malignant mesothelioma of the pleura. It is the most common type of mesothelioma and needs to be distinguished from other mesotheliomas (Table 3).

2.2.2 Clinical presentation

At the early stage of MPM, either single or multiple small nodules or plaques arise from the parietal pleura. The pleural surface becomes progressively thicker as the tumor invades the pleural cavity [35]. In the later stages of the disease, growing tumor encases surrounding organs. The tumor typically spreads through local invasion from the parietal to the visceral pleura and into the chest wall, mediastinum, diaphragm, pericardium or spinal cord [36]. Subcutaneous invasion through a percutaneous procedure tract (i.e. after biopsy, chest tube, thoracoscopy) is reported in approximately 20 % of MPM patients [37]. Distant metastasis is thought to happen only in advanced stages either via lymphatic or hematogenous tumor spread. A clinical study on 165 MPM patients found parenchymal lung metastasis in 27%, bone metastasis in 20%, peritoneal carcinosis in 24%, and additional visceral organ involvement in 15% of patients [38]. Autopsy studies have reported distant metastases in lung, liver, adrenal glands, bone, brain, spleen, or kidney in up to 55% of deceased MPM patients [39].

The tumor usually presents unilaterally, although three percent of patients have bilateral malignant disease at presentation [40]. The average time between the onset of symptoms and MPM diagnosis is two to three months [2]. The symptoms are non-specific and depend on the stage of the disease. Only a small number of patients are asymptomatic at diagnosis. Shortness of breath (dyspnea) and chest pain are the most frequent symptoms [2]. Dyspnea can be either due to pleural effusion or due to diminished respiratory movements and encasement of the lung by the tumor itself. A retrospective study focusing on clinical and radiological findings in 363 MPM patients showed that pleural effusion was present in 86% of diagnosed patients out of which 82% had reported breathlessness [41]. Chest pain was the second most common symptom at diagnosis, occurring in 68% of patients. Pain can be due to dull parietal pleural irritation or due to tumor invasion into the chest wall causing somatic or neuropathic pain [36]. Cough and hemoptysis are uncommon but possible signs of the disease. Systemic symptoms such as fatigue, malaise, anorexia, night sweats, and weight loss are typically present in the late phase of the disease.

A rare presentation of MPM can be direct tumor infiltration into the mediastinum causing dysphagia, phrenic nerve paralysis, or superior vena cava syndrome [42]. Similarly, tumor compression of the spinal cord may lead to focal neurologic deficits, and growth through the diaphragm could result in abdominal pain, nausea, and vomiting. Cardiac involvement may lead to pericardial effusion, heart failure, or arrhythmias [36].

2.2.3 Epidemiology

MPM is a rare cancer estimated to represent less than one percent of all diagnosed malignancies [43]. The incidence of MPM follows mostly the regional use of asbestos, and has been increasing steadily in the last decades worldwide [44]. Data from Cancer Registry of Norway includes incidence rates between 1970 and 2009. They found that the incidence has increased 7.8-fold in men and 6.5-fold in women in this 40-year period. The age-adjusted annual increase was 4.2% in men and 2.9% in women [45]. A similar rise in incidence has been reported in the UK and in some other European countries with peak incidences being expected in ten years [20,44]. In contrast, in the USA the age-adjusted incidence rates of MM peaked in the early 1990s, followed by a decrease [46]. Likewise, the reports from the Finnish

Cancer Registry (FCR) show that the age-standardized incidence of MM increased with small annual variation from 1953 (0.06 / 100,00) to peak year 2007 (2.23 / 100,000) and started to decrease after that (1.48 / 100,000 in the year 2017) [1]. Figure 1 shows the variability of incidence both in men and women in Finland from 1953 to 2017.

The largest European epidemiological MPM cohort reported in the literature comes from the UK, where they analyzed 8740 cases from the UK National Lung Cancer Audit between 2008 to 2012 [20]. The median age at diagnosis was 73 years with a range between 21 to 100 years of age. Eighty-three percent of the patients were male. Similar results were published in Finland including a total of 1010 MPM patients between the years 2000 – 2012 and identified from FCR: median age was 69 years (range 25 – 96 years) and 79% were male [19].

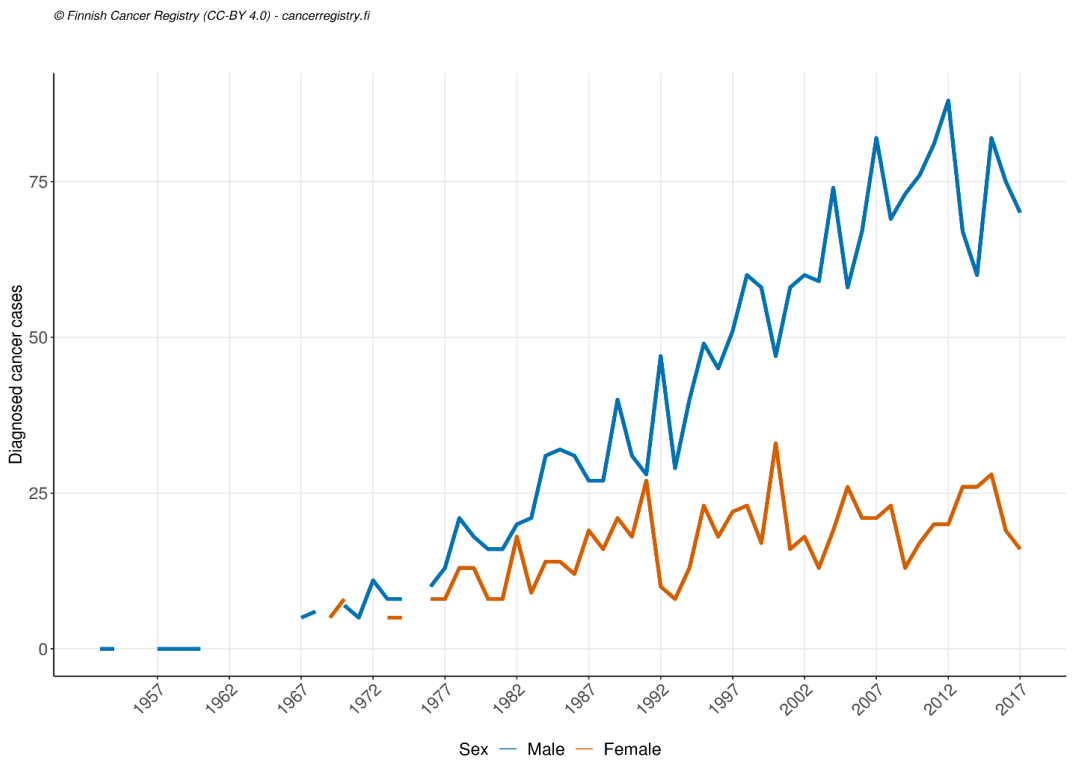


Figure 1. Malignant mesothelioma incidence in Finland from 1953 to 2017. Reproduced with permission from the Finnish Cancer Registry [1]

2.2.4 Risk factors

Asbestos exposure

Asbestos is the commercial term for naturally occurring fibrous minerals that are found in many parts of the world. Its fibrous shape, strength, and resistance are the main reasons for the global use of asbestos fibers [47,48]. Asbestos fibers are classified into two main families based on their geological origin. The amphiboles include crocidolite, amosite, tremolite, actinolite, and anthophyllite. The serpentines contain only chrysotile, which is the only asbestos type that is still widely used [49].

Asbestos exposure is the biggest risk factor for MM, and it is estimated to account for 70% to 90% of new MPM diagnoses [50]. Even a brief exposure to asbestos can result in MM. The association between asbestos exposure and MM was first described in 1960s, when Wagner et al. found a high incidence of MM in asbestos workers and among people living near a crocidolite asbestos mining area [51]. A review of 1690 mesothelioma cases found that the median latency period from exposure to disease was 32 years and over 99% of patients had a latency of over 15 years [52]. Asbestos exposure increases also the risk of lung cancer and other malignancies including laryngeal and ovarian cancer. Asbestos has also been identified as a risk factor for benign pleural diseases such as pleural plaques or exudative pleuritis, fibrosis of the lung (asbestosis) and retroperitoneum [47,53,54].

Historically, the majority of asbestos exposure has been occupational. Epidemiological analyses from France showed that the industries with the highest risks of MM among asbestos industry were shipbuilding and repairing, construction, and manufacture of metal construction materials, while the occupations at highest risk were plumbers and pipe-fitters, welders, and sheet-metal workers [55]. Finland banned the use of new asbestos in 1992. The European Union adopted a Directive which banned all remaining uses of asbestos in 2005 [56]. In addition, a national law in Finland was introduced in 2016 to reduce the risk for exposure in demolition and renovation work of old buildings. However, despite the knowledge of hazardous effects of asbestos, there is still a marked industrial use of asbestos in some parts of the world [49]. Asbestos exposure can be also non-occupational. A meta-analysis reviewed the risk of MPM in non-occupational asbestos exposure using 18 studies comprising 665 MPM patients. They found that non-occupational asbestos exposure increases the risk for MPM by 5.4-fold in household (i.e. home installation/renovation, para-occupational) and 6.9-fold in neighborhood (i.e. air pollution, industrial contamination) exposure [57].

Other risk factors

Similar to asbestos, other organic or mineral fibers can be carcinogenic and have an association with MPM [35]. For example, erionite, which is found in volcanic regions in Turkey, Italy, and USA has been linked to a high incidence of MPM [58]. In addition, carbon nanotubes have physical similarities with asbestos and are used widely in the electronic industry. They have shown *in vitro* cytotoxicity and development of MM in animal models [59]. A variety of tumors, including MPM, have been reported after either therapeutic or occupational exposure to ionization radiation [60]. There is contradictory evidence about the role of simian virus 40 in MPM; some animal and epidemiological studies have found a strong relationship for the risk of MPM but others have questioned these results and the International Agency for Research on Cancer (IARC) has classified it as not carcinogenic [61,62]. On the contrary to other thoracic cancers, no clear association between tobacco smoke and the risk of MPM has been established.

2.2.5 Pathogenesis

Asbestos induced carcinogenesis

In the 1980s, Kane et al observed that a single dose of asbestos fibers can damage the mesothelium, stimulating the recruitment of inflammatory cells to the site of the injury, which subsequently leads to mesothelioma development [63]. Inhaled asbestos fibers enter the parietal pleura through the alveoli or the lymphatic vessels of the pleural space [64]. A dose-response relationship between asbestos exposure and the risk for MPM has been proposed in epidemiological, animal, and lung tissue fiber burden studies [65,66]. However, the exact mechanism of asbestos carcinogenicity is still unknown. The hallmark of carcinogenicity of asbestos fibers is their long biopersistence and their ability to induce pleural irritation and local inflammation [67].

Currently, there are several hypotheses trying to explain the molecular mechanism of asbestos-induced pathogenesis. Firstly, the asbestos fibers themselves or the macrophages trying to digest them induce oxygen free-radicals that lead to intra-cellular deoxyribonucleic acid (DNA) damage and abnormal repair [68,69]. Secondly, asbestos fibers can tangle with chromosomes and the mitotic spindle, leading to chromosomal changes in dividing mesothelial cells [70]. Thirdly, asbestos fibers contain a variety of proteins and chemicals that cause cellular damage [68]. Fourthly, asbestos-exposed mesothelial cells and macrophages release several cytokines and growth factors that induce inflammation and increase cellular damage [71]. These markers induce activation of the nuclear factor-κB signaling pathway, which promotes mesothelial cell survival and division rather than apoptosis after asbestos exposure. Finally, dysregulated mesothelioma cells gain a growth advantage through stimulating factors such as epidermal growth factors, transforming growth factor-β (TGF-β), platelet-derived growth factor A, and angiogenic vascular endothelial growth factor (VEGF) [67]. Moreover, 90% of MMs express telomerase, which prevents telomere shortening, leading to continuous cell divisions [72].

Genetics of MPM

Normal cell growth is controlled by genetic material containing chains of DNA. Alterations in the DNA, referred to as mutations, can be either inherited (i.e. germline) or spontaneous (i.e. somatic). Somatic mutations include true spontaneous, as well as induced mutations such as those caused by asbestos. Instead of somatic mutations, germline mutations are passed from parents to offspring. In addition to germline mutations, additive somatic mutations are needed to develop an uncontrolled, cancerous, cell growth [73]. It is estimated that 5% to 10% of all cancers result from germline mutations, and certain gene defects can develop a variety of cancers known as familiar cancer syndromes [74].

The genetic landscape of MPM consists both inter- and intra-tumoral heterogeneity. The actual number of genetic mutations in cancer cells is usually low compared to other solid cancers [75,76]. Either sporadic or germline mutations of tumor suppressive genes contribute to the most common genetic changes in MPM. Tumor suppressor genes prevent cells from growing and dividing uncontrollably. The most frequently mutated gene found in MPM is *BAP1*, which encodes a nuclear deubiquitinase that regulates transcription factors and repairs double-strand DNA breaks [76,77]. Inactivation of the *BAP1* promote both genomic instability and reduced cell death, which favors malignant growth. *BAP1* mutations can be found in approximately 60% of mesotheliomas, which includes both sporadic and familial cases [75]. Other frequent inactivating mutations found in two recent and comprehensive analysis consisting almost 300 MPM cases were *NF2*, *CKDN2A*, *TP53*, *LATS2*, *SETD2*, *DDX3X*, *ULK2*, *RYR2*, *CFAP45*, *SETDB1*, and *DDX51* [76,78]. In addition, many other gene alterations have been less frequently reported [79]. Based on these findings, it is likely that several mutations are needed to accumulate for MPM development. These genetic mutations lead to several alterations in intracellular

signaling pathways which regulate cell growth, proliferation, and survival including the p53 signaling, Hippo cell cycle, DNA repair, MAPK, ribonucleic acid (RNA) helicase, histone methylation, and PI3K/AKT/mTOR pathways [78,79].

The observation that only 5% to 15% of asbestos exposed people will develop MPM, suggests that inherited factors may affect the susceptibility to asbestos exposure. The observation that MM clustered in certain families with small environmental asbestos/erionite exposure led to the finding of germline mutations in the gene encoding *BAP1* in multiple relatives with MM [80]. Subsequently, rodents that carry one abnormal copy of *BAP1* were exposed to low levels of asbestos and developed frequent and accelerated MM [81]. The defect in germline *BAP1* gene has been confirmed to also cause other cancers such as skin or uveal melanomas, renal cell carcinomas, breast carcinomas, sarcomas, and brain tumors [82]. After the initial finding of *BAP1*, several other germline cancer susceptibility mutations have been found; one study found 24 mutations in 13 genes in 198 patients (12 % of tested patients) [83]. *BAP1* was the most common mutation including 3% of tested patients, while other germline mutations were in genes *BRCA1-2, CHEK2, CKDN2A, ATM, MRE11A, TP53, MSH6, TMEM127, SDHA, VHL, WT1.* The clinical characteristics associated with germline mutations were peritoneal MM comparing with pleural disease, minimal-to-no asbestos exposure, young age, second cancer, or another MPM in the family.

2.2.6 Imaging

Imaging plays an essential role in the diagnosis, guidance of treatment decisions, and follow-up evaluation of MPM patients. Computed tomography (CT) is the primary radiological method used in the assessment of MPM, whereas magnetic resonance imaging (MRI) or positron emission tomography (PET) can also prove useful information in some cases [3]. However, MPM cannot be distinguished from other pleural malignancies solely from imaging, and the majority of the published literature concentrates on the differences between benign and malignant pleural disease in general.

Imaging modalities in MPM diagnosis

Chest X-ray is usually the first radiological examination used when pleural disease is suspected. However, its clinical benefits are narrow as it can only reveal suspicion of pleural tumor, effusion or plaques [84]. Ultrasound (US) is a high frequency sound wave. Pleural US is usually used to confirm the suspicion of pleural disease detected in X-ray. Pleural effusion aspiration or needle-biopsies of abnormal pleura are done using US-guidance [85]. Moreover, contrast-enhanced US showed sensitivity of 98% and specificity of 95% in differentiating malignant and benign pleuropulmonary lesions although this method has not been largely studied [86].

CT is an X-ray imaging method, which digitally produces three-dimensional images [87]. CT of the chest and upper abdomen is the most used technology in MPM evaluation, as it demonstrates the extent of primary tumor, its local invasion, intrathoracic lymph nodes, and extrathoracic metastasis. Intravenous contrast should be administered in order to achieve tissue enhancement, which aids in the differentiation of malignant and benign lesions. The diagnostic accuracy of CT in 370 suspected pleural malignancy patients resulted in sensitivity of 68%, specificity of 78%, positive predictive value of 80%, and negative predictive value of 65% [88]. These numbers suggest that a significant proportion of patients with pleural malignancy have no evident malignant findings in the CT scan. Pleural mass, and a focal or diffuse pleural thickening of more than 1 cm is suggestive of malignant pleural disease. Seely et al evaluated diagnostic CT scans of 92 histologically confirmed MPM patients; all patients had some form of pleural mass or thickening: costal in 97%, mediastinal in 95%, paravertebral in 92%, diaphragmatic in 76%, fissural in 72%, whereas the thickening was nodular in 79 patients (86%). Pleural effusion was found in 87% and calcified pleural plaques in 43% [40]. In addition to pleural thickening,

tumor volume evaluation either by manual or semi-automatic CT protocols have been proposed but additional data is needed for wide clinical use [89]. Modified Response Evaluation Criteria in Solid Tumors (mRECIST) criteria is mesothelioma specific and currently recommended for therapy response assessment. It combines unidimensional tumor thickness measurements at two sites in three different levels on axial CT scans into one measure. It was originally published in 2004 with a newly revised version published in 2018 [90,91].

MRI is based on the magnetization properties of hydrogen protons. The minor contrast differences between tissue lines can be effectively characterized using MRI, which makes it better to visualize soft tissue pathologies compared to CT [92]. Functional MRI, such as diffusion-weighted imaging, is a promising novel method for oncologic applications. It can be used non-invasively to quantify diffusion of water molecules in tissues. A prospective study investigated its differential ability on pleural malignancy and reported a sensitivity of 93%, specificity of 94%, and accuracy of 94% [93]. Another study showed that novel MRI applications can help to diagnose histologic subtypes of MPM, and to recognize intratumor distribution of sarcomatoid or epithelioid components [94]. However, to date, its clinical applicability has not been well defined.

PET is a form of nuclear imaging, where a positron emission from the radioactive tracers is detected by the PET scanner [95]. PET scan provides information on tissue metabolism, which differs from previously discussed modalities as they rely on anatomical changes. PET scan produces three-dimensional images but has a poor anatomic resolution, thus it is usually combined with CT for the benefits of both techniques (PET-CT). Fluorine-18-deoxyglucose (FDG) reflects glucose metabolism, and is the most used radiotracer in clinical oncology [96]. Malignant cells show increased glucose utilization due to a high amount of glucose transporter proteins and enhanced glycolysis [97]. However, FDG uptake is not specific to malignancies: an increased metabolism detected by PET scan is also reported in several benign conditions, mainly due to inflammation or infection [98]. A meta-analysis reviewed a diagnostic accuracy of PET-CT in differentiating benign and malignant pleural diseases [99]. They reviewed 16 studies with 745 patients and reported a pooled sensitivity of 95% and a specificity of 82%. Similarly, several studies have been published for diagnostic use of PET-CT in MPM. The sensitivity ranged between 88% to 92% and specificity between 75% and 93% depending on the underlying population [100,101]. A semi-quantitative analysis, called standardized uptake ratio (SUV), is frequently calculated using FDG ratios between cancer and non-cancer tissues [102]. Large differences have been reported between the mean SUV of malignant (4.9 - 9.4) and benign (0.8 -3.3) lesions. One study revealed a sensitivity of 94%, specificity of 100%, positive predictive of 100% and negative predictive value of 93%, if the SUVmax was over 2.2 [100].

2.2.7 Staging

Staging refers to the sorting cancer patients into groups according to anatomical extent of the disease. Anatomical stage is used for treatment planning and prognostic evaluation. The American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) released a new, 8th edition, staging manual for thoracic oncology in 2017 [4]. Historically, several different staging systems have been used in MPM. The first one was introduced by Butchart, including information of tumor extent in only 29 surgically treated patients [103]. Another frequently used staging method by Brigham was proposed based on the tumor, resectability, and nodal status in patients undergoing multimodality treatment [104]. In order to unify staging systems with other cancers, tumor-node-metastasis (TNM) classification was introduced by the International Mesothelioma Interest Group (IMIG) in the 1995 [105]. The IMIG staging was the first international MPM staging system that was accepted by the AJCC/UICC. The problem in these previous staging systems is that they rely mostly in single-center surgical data. Thus, the International Association for the Study of Lung Cancer (IASLC)

and IMIG launched a large international staging database using retrospective information from 1995 to 2009. However, this database lacked information on TNM-categories. Thus, for the newest 8th edition, a second IASLC clinicopathological database was created consisting of 2460 MPM patients [106]. Out of these patients, clinical staging information was available in 827, postsurgical in 830, and both in 803 cases.

In the staging process, three main features are evaluated separately: the extent of local invasion of the primary tumor (T), and presence or absence of lymph node metastasis (N) or distant metastasis (M). Two major changes were proposed in the new staging edition. Because there was no survival differences in the previous T1a and T1b categories, they were merged into single T1 [107]. In practice, this change means that no further distinctions are made if the tumor involves the parietal or visceral pleura. T3 and T4 tumors are both categorized as locally advanced with a difference that T3 tumors might still be potentially resectable, whereas T4-tumors are seen as technically unresectable (Figure 2). Similar to T-category, the survival difference was found only in patients with negative nodes versus nodal metastasis, but not between different nodal stations [107]. Thus, previous N1 and N2 stations were collapsed into new N1, which accounts for ipsilateral bronchopulmonary, hilar or mediastinal lymph nodes. Previous N3 will be reclassified as N2 including contralateral bronchopulmonary, hilar, or mediastinal and supraclavicular lymph nodes (Figure 3). There were no changes in M-category: patients are either categorized into presence or absence of distant metastasis.

After assigning either clinical or pathological T, N, and M descriptors, they are categorized into different stage groups (Table 1). Clinical stage refers to clinical and radiological information and is used to guide treatment options. Whereas pathological stage combines clinical stage with surgically obtained tissue samples and it gives a better assessment of prognosis and possible adjuvant therapy. It has been proposed that only mesotheliomas diagnosed by maximal cytoreductive surgery should be pathologically staged, whereas less-invasive samples should undergo only clinical staging [108].

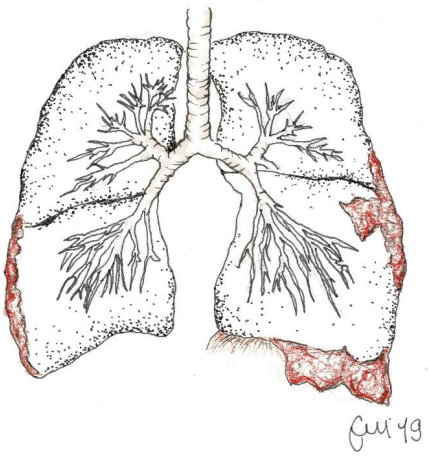
CT remains the primary method for clinical staging, although the correct clinical staging usually needs a combination of different diagnostic methods. In a comparison of MRI and CT for tumor extent evaluation, MRI was superior than CT in revealing chest wall, mediastinum or diaphragmatic muscle invasion and could lead to reclassification of up to 30% of surgical candidates [109,110]. In addition, MRI is the most accurate method for evaluating bone, cardiac, mediastinal, and central nervous system (i.e. brain or spinal cord) metastasis [111]. In nodal evaluation, a CT threshold greater than 10 mm in short-axis diameter is considered abnormal, but it is not specific for malignancy. The PET-CT has superior accuracy compared to CT for nodal metastasis assessment [111]. For occult distal metastasis, PET-CT discovers about 10% of metastasis that are not found on a CT. However, due to high false positive FDG uptake due to infectious or inflammation-related causes, invasive tissue sampling using laparoscopy, endobronchial ultrasound or mediastinoscopy may be needed, especially if surgical treatment is considered [112]. In general, the correlation with clinical and pathological stage is poor, and upstaging has been reported in up to 80% of patients with cTNM stage I or II disease [113]. Thus, the latest guidelines suggest that staging is done mainly by contrast enhancement CT, but if radical surgery is considered, more intensive investigations should be done in order to define the precise clinical stage and inform appropriate treatment choices [8,9].

T1

Involves ipsilateral pleural surfaces only:
- parietal
- visceral
- mediastinal
- diaphragmatic

T2

Involves ipsilateral pleura with invasion into:
- lung and/or
- diaphragmatic muscle



T3

Involves ipsilateral pleura with invasion at least one site:
- endothoracic fascia
- chest wall (solitary focus extending into soft tissue)
- mediastinal fat
- pericardium (non-transmural)

T4

Involves ipsilateral pleura with at least one of the following features:
- diffuse invasion of the chest wall
- direct extension to the contralateral pleura, spine, peritoneum, or mediastinal organs
- transmural invasion of the pericardium or myocardium

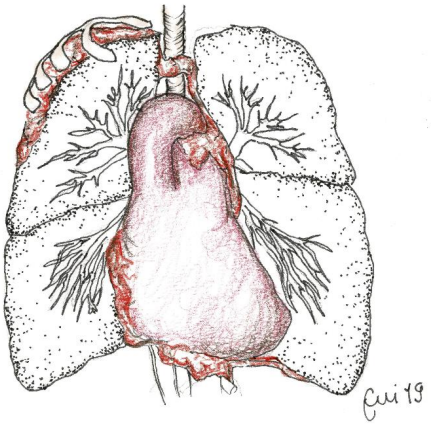
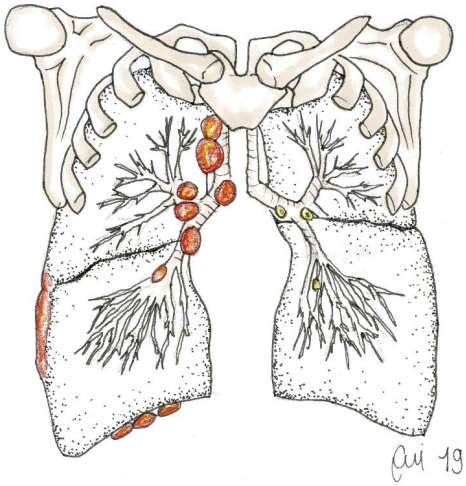


Figure 2. Malignant pleural mesothelioma staging, T-stages [4]

N1

Metastases to ipsilateral intrathoracic lymph nodes



N2

Metastases to ipsilateral or contralateral supraclavicular lymph nodes

OR

metastases to contralateral intrathoracic lymph nodes

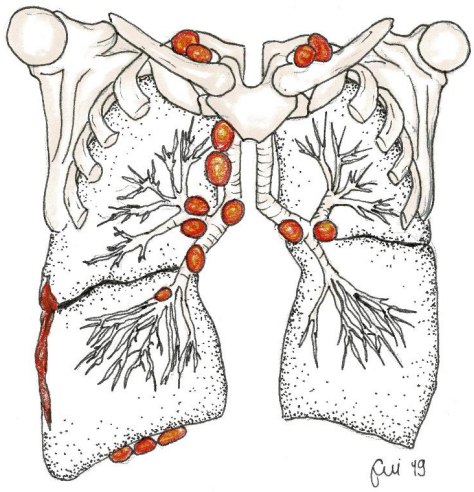


Figure 3. Malignant pleural mesothelioma staging, N-stages [4]

Table 1: TNM stage with proportional prevalence according to "the best" overall stage (n = 2414); modified from AJCC/UICC Cancer Staging Manual 8th edition [4]

Stage with prevalence (%)	T-category	N-catecory	M-category
Stage IA (15%)	T1	N0	M0
Stage IB (37%)	T2, T3	N0	M0
Stage II (11%)	T1, T2	N1	M0
Stage IIIA (13%)	T3	N1	M0
Stage IIIB (20%)	T1, T2, T3	N2	M0
	T4	Any N	M0
Stage IV (4%)	Any T	Any N	M1

TNM, tumor, node, metastasis; AJCC, American joint committee on cancer; UICC, union for international cancer control. The best stage is defined as pathological stage if available, otherwise as clinical stage.

2.2.8 Circulating biomarkers

Circulating biomarker refers to measurable molecules from the bloodstream that reflect normal biological pathways, pathogenic processes, and/or response to an exposure or intervention. Ideally, a biomarker for cancer diagnosis should distinguish those with a specific type of cancer from those who do not have cancer. Further, it should also have high stability in the blood with simple, accurate, and reproducible measurement method [114]. Many different circulating tumor markers have been studied in relation to diagnosis, disease monitoring, prognosis, or screening of mesothelioma [115]. Previous studies have demonstrated that blood levels of mesothelin or soluble mesothelin related peptides (SMRP), High Mobility Group Box 1, megakaryocyte potentiating factor (MPF), osteopontin, or fibulin-3 are elevated in MPM compared to asbestos-exposed or secondary pleural malignancies [115–117]. SMRP has the most evidence in the context of diagnosis or disease monitoring. A meta-analysis reviewed 30 publications for the use of SMPR for diagnosis [116]. It showed a pooled sensitivity of 61% and a specificity of 87%, with and area under the curve of 0.81. However, only 16% to 40% of asbestos-exposed individuals who develop mesothelioma will have elevated SMRP levels on follow-up. In addition, SMPR have been shown to positively correlate with tumor size, stage, and falling after surgery in patients with positive chemotherapy response [118]. The original publication on fibulin-3 showed intriguing diagnosis accuracy, but validation cohorts haven not confirmed these findings: sensitivity of 100%, specificity of 94% with and AUC of 0.87 [117,119]. However, as stated in systematic review in 2017, more data is needed and none of these markers are currently recommended in routine clinical use [7].

Activins and follistatins

Activins are dimeric proteins that belongs to the TGF- β family [120]. They contain two β-subunits (βA, βB, βC, βD, and βE) generating homo- or heterodimers. The most studied and biologically active isoforms are activin A (βA and βA), activin B (βB and βB), and activin AB (βA and βB) [121]. Like other members of the TGF- β family, activin signaling is mediated through the transmembrane serine/theorine kinase receptor type I and II (ActRI, ActRIIA/ ActRIIB) [122]. The most common signaling pathway starts when activins binds to the ActRII which triggers ActRI, subsequently leading to the phosphorylation of Smad2/3 [123]. In addition to the Smad-mediated signaling pathway, other

cascades are recognized with different biological actions [15,124,125]. Activin signaling is controlled by several biological antagonists; inhibins, betaglycan, cripto, and BAMBI counteract activins at a cell membrane level, while follistatin (FS) and follistatin-like 3 (FSTL3) prevent activin signaling by binding and forming inactive complexes [126–128]. The ability of FS and FSTL3 to bind and neutralize activin A is approximately 10-fold higher compared to activin B [129]. Similar to activins, FS and FSTL3 also antagonize other members of TGF-β family called myostatin and several bone morphogenetic proteins [130].

Activins were originally isolated from gonads and identified as inducers of follicle-stimulating hormone [121]. Now, they are known to be widely expressed throughout the body and to have many biological functions such as regulating reproduction and embryogenesis, cutaneous wound healing, hematopoiesis, tissue homeostasis, hormonal secretion, immune responses, angiogenesis and to control cellular proliferation, differentiation, and apoptosis [131–136]. In healthy people, serum activin A levels increase with age [137]. No differences were observed between sexes but elevated values were observed in women during gestation or menstrual cycles [138].

Increased activin expression has been linked with liver, pancreatic, kidney, and lung fibrosis [139–142]. A number of in vitro and clinical studies have found that activin levels are elevated during systemic inflammation such as septicemia, and inflammatory diseases like rheumatoid arthritis or inflammatory bowel diseases [132,143,144]. Activins can lead to anemia via reduced erythropoietin synthesis [145]. In addition, elevated activin A levels are measured in thyroid diseases, chronic renal failure, chronic obstructive pulmonary disease, and metabolic syndrome [137,146,147].

Depending on the tissue or stage of the tumor, activins can either inhibit or stimulate cell growth. For example, growth suppressive effects have been reported in breast, pancreatic and low-grade prostate cancer cells [148,149] whereas activin overexpression has been linked to more aggressive behavior in myeloma, high-grade prostate, lung, oral, esophageal, and colorectal cancers [150–155]. Moreover, high circulating activin A levels have been linked to worsened survival in myeloma, breast, prostate, lung, esophageal and pancreatic cancers patients [154–157]. Thus, it is postulated that activins are potent negative growth regulators in normal conditions and early stages of tumorigenesis, but several mutations in activin signaling genes leads to cancerous growth which is exerted by activins [158,159]. Furthermore, activins are hypothesized to indirectly contribute to cancer progression by modifying the tumor microenvironment through regulating angiogenesis and inflammatory responses [160].

FS was first isolated from bovine follicular fluid [126]. It is a single-chain glycosylated protein, with three major isoforms, FS288, FS315, and FS303 [161]. The structure of FSTL3 (also known as follistatin-related protein, FSRP) is similar to FS, except for the lack of one FS-domain and heparin binding sequence [162]. Activin regulation is the main biological function of FS and FSTL-3. Follistatins' importance in embryogenesis was noted when a variety of lethal defects were observed in FS-knockout mice [163]. Like activins, FS and FSTL3 can have a dual role in tumorigenesis. In vitro studies have showed that adding FS to ovarian tumor cells slowed the growth rate of tumor cells, whereas prostate cell lines showed overproduction of FS which accelerated tumor growth [164]. In clinical studies, elevated FS or FSTL3 levels have been linked to poor survival in hepatocellular, ovarian, gastric, and breast cancer [165,166].

Activins in MPM

An original report on activins in MPM was published in 2012, where it was found that activin subunits βA, βB, and βC are upregulated in MPM cell lines [11]. Activin A was overexpressed in tumor tissue compared to normal mesothelium, and most tumors stained IHC positive for activin A. In addition,

inhibition of activin A led to suppressed tumor growth. They observed no upregulation of FS or FSTL3. Another study found both activin A and B to be overexpressed in MPM tumor tissue and in cultured cell lines [10]. Soluble ActRIIB-blockage reduced migration and invasive growth via ERK or cyclin D pathway signaling. In contrast to activins, they found that expression of FS and FSTL3 were inconsistent among tumor cells, with FSTL3 levels mostly upregulated and FS downregulated. One multi-institutional clinical study on circulating activin A in MPM has been previously published [12]. They collected plasma samples from 129 MPM patients, as well as 45 healthy controls and 16 non-malignant pleuritis patients. Circulating activin A levels were elevated in MPM compared to healthy controls but were similar to those of pleuritis patients. In MPM patients, significant differences were reported based on tumor histology with the lowest values being observed in the epithelioid subtype. In addition, they found activin A to be positively correlated with pretreatment tumor size and low activin A levels to be associated with longer survival after adjustments for age, histology, sex, and TNM stage.

Activins and cancer cachexia

The international consensus report defined cancer cachexia as a multifactorial syndrome consisting of ongoing loss of muscle mass, with or without loss of fat mass, that cannot be fully reversed by nutritional support [167]. Cachexia is associated with physical impairment, reduced quality of life, impaired tolerance to anticancer therapy, and increased mortality. The prevalence of cachexia in advanced cancer patients is estimated to be 60% to 80% depending of the cancer tissue [168]. The degree of cachexia is defined by the degree of weight loss, symptoms and clinical signs: precachexia (weight loss <5%, early metabolic signs), cachexia (weight loss > 5% with or without low BMI or skeletal muscle mass) and refractory cachexia (preterminal cancer with active catabolism, PS score 3 or 4, and life expectancy < 3 months) [167].

A key component in cachexia is catabolism caused by tumor- or host-mediated cytokines and systemic inflammation. Pro-inflammatory cytokines, such as tumor necrosis factor α, interferon-γ, interleukin (IL) 1 or IL6, or C-reactive protein (CRP) trigger muscle wasting and are upregulated in preclinical cachexia models [169]. However, findings for inflammation markers in cachectic cancer patients are controversial and depend on tissue site and stage of the disease [170,171]. Other markers of cachexia include growth differentiation factor (GDF) 8 (myostatin) and 15, ghrelin, testosterone, insulin-like growth factor 1, zinc alpha 2-glycoprotein, leptin, and parathyroid hormone release peptide [172,173]. In addition to molecular mediators of cachexia, direct tumor burden, immobility, or treatments may lead to reduced food intake, impaired muscle mass, and weight loss [173].

The first evidence that activins play a role in cancer cachexia was found on inhibin-deficient mice, that developed gonadal tumors producing 10-fold activin A and B levels, which were observed to lead to lethal wasting-syndrome [174]. Myostatin, another TGF- β protein, is predominantly secreted from muscle cells and acts as a negative regulator of muscle growth [175]. It shares, as well as GDF-11, a common signaling pathway with activins [176]. Multiple mouse models have showed that blocking ActRII prevented muscle wasting, and prolonged survival without affecting tumor growth or inflammatory cytokine levels [14]. Similar effects were seen in myostatin-deficient mice, which suggests that other ligands also contribute to muscle wasting [177]. Blocking of the activin/myostatin pathway has been studied in several phase I-II clinical trials, with positive results in muscle volume and strength [178]. In addition, one clinical study has compared circulating activin and myostatin levels on lung and colorectal cancer patients [13]. Overall, these studies have shown an association with activin levels and cachexia, whereas myostatin levels have been shown to correlate with muscle mass but with not cachexia. These observations are comparable to those obtained in animal models.

2.2.9 Diagnosis

A definite diagnosis of MPM requires both morphologic and immunohistochemical (IHC) features suitable for MPM as well as a histopathological confirmation of invasive tumor growth into adjacent tissues [17]. However, in some instances, a reliable diagnosis can be achieved if clinico-radiological findings are consistent with MPM and with a supporting cytological sample. However, there are several diagnostic pitfalls in the cytological specimen, which makes cytological diagnosis less reliable [179]. Pleural effusion in MPM is often hemorrhagic or inflammatory, which might mask malignant cells. Obtaining a cellblock from effusion samples allows ancillary methods, such as IHC or molecular analysis to be done. These methods are needed to distinguish malignant from reactive mesothelial proliferation [180]. Once the malignant nature has been verified, the next step is to demonstrate the mesothelial subtype of the tumor cells, which is most often done by IHC staining (Table 2). In addition, the sarcomatoid mesothelioma will not exfoliate sufficient tumor components in the pleural effusion, which makes it impossible to diagnose solely from cytological specimen [16].

Reaching a conclusive MPM diagnosis from histological samples can also be challenging, especially from small biopsies. In France, a national pathologist panel reviewed over 1000 cases with initial mesothelioma diagnosis in 1998-2003; they confirmed the diagnosis in 67% of the cases, ruled out 13%, and classified the rest as uncertain [55]. Similar results have been published afterwards, even if diagnostic methods have been improved. Another study compared biopsy size with diagnosis and found that biopsies over 10 mm achieved definite diagnosis in 86% of cases, while those smaller than 10 mm were classified as definite only in 14% of cases [181]. Also, needle biopsies are associated with misclassification of MPM subtype in up to 44% of cases [182]. Therefore, thoracoscopy, either medical pleuroscopy or video-assisted thoracic surgery (VATS), is the recommended diagnostic procedure [9]. The benefits of surgical operation are direct visual observation of the pleura, improved size of the tissue samples, and possible pleural effusion treatment in the same procedure. The complication rate in diagnostic VATS is less than 10% with most reported complications being local infection, small hemorrhage, subcutaneous emphysema, and a mild fever [183]. In some instances, especially if thoracoscopy is unsuitable, either US- or CT-guided transthoracic needle biopsy can be carried out at the expense of the diagnostic sample size.

Histological classification of MPM is important for prognostic evaluation and treatment guidance. The World Health Organization (WHO) released a new classification of tumors of the pleura in 2015 (Table 3) [17]. Histologic classifications remain unchanged compared to 2004 with the following main three histologic subtypes being recognized: epithelioid, biphasic, and sarcomatoid [184]. Desmoplastic mesothelioma is a rare sarcomatoid variant, which represents approximately 2% of all MPM cases. In contrast, epithelioid is the most common subtype comprising approximately 60% of MPM cases [35]. Histological tumor grade describes the appearance of tumor cells and tissue under the microscope. It indicates the degree of tumor differentiation. Well-differentiated tumors present close to normal cells or tissue and tend to have more indolent behavior. Thus, the purpose of applying a grading system in MPM would be to allow differentiation of indolent tumors from more aggressive ones. Although there have been several studies regarding mesothelioma grading, to date, no uniform grading system is recommended by WHO or other organizations [17].

MPMs histological subtypes have a wide histopathological heterogeneity. Epithelioid MPM comprise polygonal, oval or cuboidal tumor cells with, in general, ample cytoplasm and round nuclei with prominent nucleoli [16]. A variety of morphological patterns are recognized within the epithelioid subtype, such as tubulopapillary, papillary, micropapillary, trabecular, solid, and pleomorphic patterns [185]. The presence or absence of mitoses, acinar structures, myxoid change, psammoma bodies, necrosis, cytologic features (microcystic, clear cell, deciduoid, small cell) can present with various

degrees in epithelioid mesotheliomas [35]. The sarcomatoid subtype consists spindle-shaped (greater than two times in length than width) cells characterized by elongated nuclei, numerous mitosis, and eosinophilic cytoplasm. The histological features that can be recognized in sarcomatoid tumors include desmoplastic, lymphohistiocytoid, and osteoid- or chondrosarcomatous differentiation [186]. Sarcomatous tumors can also resemble other soft tissue sarcomatous elements [184]. Desmoplastic MPM is characterized by dense collagenized tissue separated by atypical cells in a storiform pattern [187]. MPM is classified as biphasic (or mixed), if the tumor contains at least 10% of both epithelioid and sarcomatoid patterns [184].

Immunohistochemistry is used to visualize the tumors antigen expression using antibody-antigen-interaction and it is widely used in cancer diagnostics [188]. IHC staining is an important supplementary technique in discriminating MPM from other benign or malignant pleural diseases as well as differentiating MPM histological subtypes. The role of IHC depends on the morphological features of MPM as well as possible differential diagnosis [189–191]. When interpreting IHC-findings, both the localization of the stain and the number of cells staining positively are important. However, none of the IHC stains are diagnostic in all cases. Thus, the latest guidelines for pathological diagnosis of MPM suggest using panels with both positive markers for mesothelioma and markers for other malignancies that are typically negative for mesotheliomas [16]. The most widely used IHC-markers and their diagnostic prevalence are presented in Table 2. In a case with no evident discrepancies, at least two positive mesothelioma markers and two carcinoma markers are considered to be adequate for diagnosis. Additional, more specific markers, should be used when conflicting differential diagnosis are being considered [16]. For example, the loss of *BAP1* expression was investigated in a study consisting 258 malignant effusions [192]. They reported that *BAP1* protein was lost in 46 of 53 (87%) in MM and 4 of 205 (2%) carcinoma cases yielding to sensitivity of 87% and specificity of 98%.

In addition to these classical histopathological tests, newer molecular techniques have been identified in order to aid diagnosis. Fluorescence in situ hybridization (FISH) assay for the p16/CDKN2A homozygous deletion can be used to distinguish benign from malignant pleural proliferation [180]. Micro-ribonucleic acids (miRNAs) are short non-coding RNAs that regulate essential cellular mechanisms post-transcriptionally [193]. In addition to their role in normal biology, their aberrant expression in cancers significantly contributes to malignant growth, which makes them potential biomarkers. Several miRNAs have been studied from pleural and tumor tissue, pleural effusion, and peripheral blood, and preliminary findings suggest that they might have potential diagnostic value but more prospective validation is needed [194]. In addition, a sequential combination of binary gene expression ratio tests from tumor tissue was able to distinguish MPM from other common thoracic cancers and normal pleura as well as to identify histological subgroups in MPM [195].

Table 2. Antibodies used in immunohistochemical staining for differential diagnosis

Type of tumor	Positive markers (expression %)	Reference
Epithelioid MPM	Calretinin (>95) Cytokeratin 5, or 5/6 (75-100) WT1 (70-95) Podoplanin, D2-40 (90-100)	[189,190]
Sarcomatoid MPM	Cytokeratins (93) Vimentin (91) Calretinin (31)	[186]
Lung adenocarcinoma	Claudin 4 (>95) MOC31 (95-100) CEA (80-100) BER-EP4 (95-100) TTF-1 (75-85) Napsin A (80-90)	[189]
Lung squamous carcinoma	p40 or p63 (100) Claudin 4 (95) MOC31 (95-100) BER-EP4 (85-100)	[191]
Solitary fibrous tumor	CD34 (95) STAT6 (98)	[196]

WT1, Wilms tumor-1; CEA, carcinoembryonic antigen; TTF-1, thyroid transcription factor-1

2.2.10 Differential diagnosis

Differential diagnosis includes the separation of MPM from other primary pleural tumors, pleural metastasis, benign reactive mesothelial proliferation, inflammatory reactions, and other more indolent mesotheliomas [184]. In most cases, morphological features along with appropriate IHC and clinical behavior are sufficient for a correct diagnosis. However, in some instances, additional work-up is needed.

The most common differential consideration in epithelioid MPM is the metastatic non-small-cell lung carcinoma (NSCLC). They represent approximately 40% of metastatic pleural cancers [197]. Other common secondary malignancies of the pleura are breast carcinoma, malignant melanoma, gastrointestinal cancers, renal cell carcinoma, and pleural dissemination of thymoma [16]. The sarcomatoid MPM may be difficult to distinguish from some sarcomas or other tumors with sarcomatoid histology [16]. In addition, the histological distinction between the desmoplastic MPM and benign fibrous pleuritis can be challenging.

Besides mesothelial tumors, the WHO classification of tumors of the pleura recognizes primary pleural mesenchymal tumors and lymphoproliferative disorders (Table 3) [184]. Mesenchymal tumors are further divided into three groups: fibroblastic tumors, vascular tumors, and tumors of uncertain differentiation. Rarely, lymphomas can arise primarily from the pleural surface [198]. Most cases are associated with immunosuppression or chronic pleural inflammation and they can present with no

evident tumor mass; the diagnosis is made with flow cytometry, molecular examination along with IHC. More commonly, a pleural involvement can coexist with generalized hematologic malignancy [199].

The existence of an in-situ form of mesothelioma is debated. The latest pathological classification suggests the use of the term "atypical mesothelial hyperplasia" since there is no reliable method to distinguish possible in situ phase from reactive proliferation [17]. More recent expert proposal suggested that mesothelioma in situ could be diagnosed in the absence of clinical or radiological evidence of tumor with mesothelial proliferation limited to serosal surface and IHC/molecular findings consisting of MM [108]. In-situ MM or atypical hyperplasia may show high cellularity, numerous mitoses, necrosis, cytologic atypia, and formation of papillary structures [180]. The presence of invasion is the single most useful criteria for malignant growth. In addition, several genetic markers have been studied for separating benign from malignant mesothelial processes [200]. The loss of *BAP1* in IHC or deletion of *p16/CDKN2A* by FISH are the most promising markers, since they have been reported only in malignant diseases [201]. However, the low sensitivity (approximately 50%-80% in both) and the lack of specificity for MPM (both can be found in other malignancies as well) needs to be considered.

The term 'diffuse malignant mesothelioma' is used to separate it from other distinct entities of mesotheliomas. Identical histopathologic, IHC, and ultrastructural features compared to diffuse MPM have been reported in localized malignant mesothelioma (LMM) [184]. These solitary circumscribed tumors present with profound invasion without diffuse pleural spread of the tumor. The diagnosis requires histology comparative with MM as well as a correlation with imaging and surgical findings to ensure that there is no diffuse component. Surgery can be curative in LMM, but recurrence or metastasis can occur after resection. The largest surgically treated series published so far reported that 10 out of 21 patients were disease-free after a median follow-up of 4.8 years (range: 1.5 to 11 years) [202]. Another study looked at previously published LMM cases (n = 48) and reported a pooled median OS of 36 months with a range of 0 to 132 months [203]. Well-differentiated papillary mesothelioma (WDPM) is a rare variant that usually involves the peritoneum in young women [204]. When found on pleura, it is characterized by exophytic papillary architecture with broad fibrovascular cores. They usually present with superficial growth with no or only minimal areas of invasion [205]. These tumors have indolent behavior, with a reported median OS of 74 months [204]. Adenomatoid tumors are benign mesothelial neoplasms that mostly arise from the genital tract but can rarely be found on pleura, peritoneum or pericardium [184].

Table 3. World Health Organization classification of tumors of the pleura [17]

Mesothelial tumors	Lymphoproliferative disorders	Mesenchymal tumors
Diffuse malignant mesothelioma	Primary effusion lymphoma	Vascular tumors
Epithelioid mesothelioma	Diffuse large B-cell lymphoma	Angiosarcoma
Sarcomatoid mesothelioma	associated with chronic inflammation	Epithelioid hemangioendothelioma
Desmoplastic mesothelioma		Fibroblastic tumors
Biphasic mesothelioma		Solitary fibrous tumor
Localized malignant mesothelioma		Malignant solitary fibrous tumor
Epithelioid mesothelioma		Calcifying fibrous tumor
Sarcomatoid mesothelioma		Desmoid-type fibromatosis
Biphasic mesothelioma		Uncertain differentiation
Well-differentiated papillary mesothelioma		Synovial sarcoma
Adenomatoid tumor		Desmoplastic round cell tumor

2.3 Survival and prognostic factors in MPM

Survival time is usually calculated either from the time of diagnosis or study enrollment until death, cancer recurrence or the end of study follow-up [206]. Prognostic factors are variables obtained from the tumor or patients that are available at the time of the diagnosis. They are related with the natural history of the disease, in the absence of therapy, and are associated with overall survival (OS) or disease-free survival (DFS). Prognostic factors may be needed in order to choose the appropriate treatment strategy for individual patients as well as to design or conduct a clinical trial [207]. In most malignant tumors, prognosis is influenced by a variety of variables called covariates. In order to determine if one factor affects prognosis independently of others, multivariate analyses are performed.

2.3.1 Statistical survival analyses

The most common endpoints in cancer trials are OS, DFS and progression-free survival (PFS), response rate (RR), and quality of life (QoL). Survival analyses are usually performed either by univariate cox regression or Kaplan-Meier method, which provides a visual cumulative survival probability curve [208]. The comparison between different survival curves is usually tested by log rank test, which weighs the effects of differences observed throughout follow-up [208]. The cox proportional hazards model is the most commonly used multivariate analysis, which is used to adjust for one or more covariates [209]. Survival data is usually presented as hazard ratios (HRs), which reflects to event rate at the given time, with 95% confidence intervals (CIs), [206].

2.3.2 Mortality

Following the rise in the incidence of MPM, age-adjusted mortality rates are estimated to have increased by 5.4% per year worldwide between 1994 and 2008. Analyses from the WHO mortality database estimated over 38,000 annual deaths globally [210]. The overall median survival reported from large international register studies varies from 9 to 12 months [20,211]. Likewise, the median survival of Finnish patients was 9.7 months in the FCR analyses [19].

2.3.3 Clinical prognostic factors

Table 4 reviews studies considering clinical prognostic factors. Different study designs and inclusion criteria need to be taken account when assessing survival and prognosis. The most consistent independent markers of good prognosis in large population-based registry studies are young age, epithelioid histology, female sex, and good performance status (PS) [20,23,106]. After multivariate adjustments, many studies have shown that the presence of symptoms such as chest pain or weight loss are associated with a higher mortality [212]. Prior asbestos exposure is considered more of a risk factor than a prognostic factor, although some studies have reported better prognosis for patients with no or minimal asbestos exposure [213]. Similarly, one study found a 2.6-fold longer survival in patients who developed MPM after lymphoma-related radiation therapy, when compared to asbestos-related patients [214].

In surgically treated patients, predictors of longer survival are epithelioid subtype, adjuvant chemotherapy, negative surgical resection margins, and absence of lymph node metastasis [104,215]. Moreover, in a three-year survival analysis after following curative intent surgery, clinicopathological features associated with prolonged survival were young age, female sex, epithelioid subtype, normal preoperative white-blood cell, hemoglobin, and platelet counts [22].

Several prognostic scores have been created by combining different variables to give a better survival estimation [212,216,217]. The best validated ones are from the Cancer and Leukemia Group B and European Organization for Research and Treatment of Cancer, both of which were produced from phase II clinical trials, which limits the generalizability to the overall MPM population. In contrast, one study reported an unselected MPM cohort of 482 patients and developed a prognostic tree, which was validated in a separate cohort [212]. They showed that the combination of weight loss, ECOG PS, Hb, albumin, and histology was able to stratify patients into four different risk groups. The single strongest prognostic variable was weight loss, and the best prognosis was found for patients with no weight loss, Hb over 153 g/l, and normal albumin levels (group 1; median survival of 34 months comparing to 7.4 months in group 4). The same prognostic model could also stratify surgically treated patients into risk groups according to survival: median survival in group 1 was 82.5 months compared to 22.2 months in group 4 [218]. A prognostic score has also been proposed for patients undergoing multimodality treatment [219] and it includes pre-chemotherapy tumor volume, progressive disease after induction chemotherapy, pre-chemotherapy CRP, and histological subtype. The original cohort reported a median survival of 34 months on patients with 0-points, compared to 4 months in patients with 4-points – these findings have been also externally validated in an independent cohort.

Table 4. Review of studies on clinical prognostic factors in MPM

Country [Ref]	Published / Data collected (years)	Population: N / male % / age(years)	Study design	Inclusion criteria	OS (months)	Independent prognostic factors (comparison variable)	Effect size (95% CI or p-value)
UK [20]	2015/ 2008-2012	8740 / 83% / 73	Prospective follow-up cohort, NLCA	Population-based: Histopathological or clinic-radiological proven MPM	9.5	PS 4 (PS 0) Sarcomatoid (epithelioid)	6.49 (5.24-8.05) 2.40 (2.16-2.70)
USA [23]	2010/ 1973-2006	9701 / 81% / 72	Retrospective follow-up cohort, SEER registry	Population-based: MPM diagnosis	12	Age (cont.) Sex, male (female) Non-epithelioid (epithelioid) High tumor grade (low) Local SEER stage (distant) Surgery, yes (no)	1.02 (1.01-1.04) 1.32 (1.01-1.72) 1.15 (0.86-1.53) 1.55 (1.36-1.76) 0.84 (0.55-1.29) 0.62 (0.48-0.79)
International [106]	2014/ 1995-2009	2141 /80% / 50-65	Retrospective follow-up cohort, IASLC database	Diagnosis of MPM with complete dataset available	NA	pTNM stage IV (stage I) Non-epithelioid (epithelioid) Sex, male (female) Age > 50 (age < 50) Palliative surgery (radical) Adjuvant treatment, no (yes) PLT > 400 (< 400) x 10^9/l WBC > 15.5 (<15.5) x 10^9/l	2.49 (0.0001) 1.80 (<0.001) 1.70 (0.0006) 1.61 (0.0120) 1.67 (0.0008) 1.70 (0.0002) 1.50 (0.0004) 2.39 (0.0007)
International [220]	2009/ 1984-2003	523 /83% / 58	Composite analysis of 10 prospective phase II trials	Histologically or cytologically proven MPM, chemotherapy or radiotherapy naïve, PS 0-2	9.1	PS 2 (PS 0) Non-epithelioid (epithelioid) cTNM stage IV (stage I-III)	2.89 (NA) 1.60 (1.20-1.71) 1.49 (1.17-1.91)
Italy [221]	2014/ 2005-2011	241 /64% / 75	Retrospective case series, multicenter	All patients aged over 70 years with MPM diagnosis	11.4	Non-epithelioid (epithelioid) Age > 75 (age < 75) cTNM stage III-IV (stage I-II) CCI > 1 (CCI 0)	2.32 (1.66-3.23) 1.44 (1.08-1.93) 1.47 (1.09-1.98) 1.38 (1.02-1.85)
USA [222]	2007/ 1990-2005	945 /80% / 66	Retrospective case series, single center	Biopsy-proven MPM	12.5	Surgical treatment, yes (no) Non-smokers (smokers) Sex, female (male) Chest pain, no (yes) Epithelioid (non-epithelioid) Laterality, left (right)	0.75 (0.62-0.91) 0.77 (0.61-0.97) 0.63 (0.51-0.77) 0.77 (0.69-0.91) 0.59 (0.46-0.71) 0.84 (0.72-0.98)
USA [223]	2013/ 2000-2010	170 /71% / 73	Prospective, case series, single center	Surgically obtained histologically proven MPM	12.0	Non-epithelioid (epithelioid) Age (cont.) CRP, 3-50 (CRP <3) mg/L WBC > 12 (WBC<12) x 10^9/l	2.76 (1.50-5.08) 1.05 (1.01-1.08) 2.28 (1.18-4.42) 2.28 (1.22-4.25)
Turkey [224]	2009/ 1991-2008	235 /NA / NA	Retrospective, case-control according to treatment schedule, single center	Diagnosis of MPM	10.0	1)BSC group: KPS >70 (KPS<70) Non-epithelioid (epithelioid) TNM stage III-IV (stage I-II) LDH >500 (LDH <500) IU-1 2)Chemotherapy group: KPS >70 (KPS <70) 3) Surgical group: Laterality, right (left) Non-epithelioid (epithelioid)	 3.83 (1.98-7.42) 1.86 (1.06-3.26) 2.28 (1.24-4.17) 2.21 (1.19-4.09) 4.78 (2.73-8.37) 4.53 (1.23-16.7) 4.55 (1.02-20.3)
USA [104]	1998/ 1980-1997	183 / 77% / 57	Retrospective case series, single center	Trimodally treated histologically proven MPM	19.0	Non-epithelioid (epithelioid) Positive resection margins (neg.) Positive extra pleural node metastasis (neg.)	3.0 (2.0-4.5) 1.7 (1.2-2.6) 2.0 (1.3-3.2)
UK [215]	2014/ 2000-2012	252 / 86% / 59	Retrospective case series, single center	Surgically treated epithelioid/biphasic, survival longer than 90 days	18.2	Age < 60 (age >60) Epithelioid (biphasic) Negative node metastasis (pos.) Chemotherapy, no (yes)	0.70 (0.51-0.94) 0.56 (0.39-0.79) 0.67 (0.49-0.90) 1.90 (1.39-2.54)

Ref, reference; NLCA, national lung cancer audit; MPM, malignant pleural mesothelioma; PS, performance score; SEER, surveillance, epidemiology, and end results; IASLC, international association for the study of lung cancer; NA, non-available; TNM, tumor node metastasis; PLT, platelet count; WBC, white blood cell count; CCI, Charlson comorbidity index; ASA-score, American Society of Anesthesiologists; BMI, body mass index; KPS, Karnofski performance score

2.3.4 Somatic and germline mutations

The prognostic role of the most common mutation in MPM, *BAP1*, is controversial. One study found that loss of *BAP1* detected by IHC was an independent negative prognostic factor after multivariate adjustments, but other reports have not found clear survival differences [76,225]. Deletion of the *CKDN2A* gene, which encodes the well-established tumor suppressor p16, is another frequently reported genetic abnormality, and homozygous deletion detected by FISH or loss of expression detected by IHC, have both been associated with a poor survival [226]. In addition to these two most common mutations, several other genes are overexpressed in MPM with different prognostic characteristics and possible therapeutic implications [79]. A four-gene ratio test has been created with a good predictive value for OS in surgically treated patients: "good risk" patients had median OS of 16.8 months compared to 9.5 months in "poor risk" patients [227]. In addition, multiplatform molecular profiling has been used to form different clusters with prognostic significance independently of histological subtype or *CKDN2A* status [76]. The poor prognosis cluster had a high score for epithelial-mesenchymal transition, enrichment of *LATS2* and *CKDN2A* mutations, and upregulation of the PI3K and mTOR signaling pathways. Similarly, four molecular clusters established via RNA-sequencing, namely sarcomatoid, epithelioid, biphasic-epithelioid, and biphasic-sarcomatoid have been identified [78]. These genomic clusters correlated with survival better than traditional histological subclassifications. Interestingly, only 38% of epithelioid histological tumors were defined as genetically epithelioid, which underlies the heterogeneity within the MPM tumors. Patients with epithelioid histology and epithelioid genetic cluster had better survival compared to those with epithelioid histology and other clusters (HR 2.5, 95% CI 1.6-3.8).

The original finding that patients with germline *BAP1* mutation have a better prognosis derives from a series of 23 MM patients with a median survival of 5 years [228]. Similarly, another study reported a prolonged median survival of 9 years in 36 patients with germline mutations other than *BAP1*. Another study evaluated the prognostic value of germline mutations in a cohort of 239 MPM patients and found an almost four times higher median survival in patients with a germline mutation [229]. Moreover, a recent study observed that the loss-of-function mutation in any DNA repair gene was associated with improved OS after platinum-based chemotherapy [229].

MicroRNAs are involved in numerous cellular processes and may behave either as tumor suppressor or oncogene. Several different circulative miRNAs with different prognostic utility have been identified in MPM. Kirschner et al. constructed a score of six microRNAs that was able to predict over 20 months OS with an accuracy of 92% in patients with surgical treatment [230]. Moreover, a different miRNA signature could predict histological subtype with correlation with survival [231]. However, additional research is needed prior to clinical use, since most of the data is based on small sample sizes or in vitro findings.

2.3.5 Prognostic circulating biomarkers

Several routinely used laboratory measurements are reported to have a prognostic role in MPM. The best validated ones include elevated numbers of WBC, platelet counts, CRP levels, lactate dehydrogenase levels as well as high neutrophil to lymphocyte ratio, whereas low levels of albumin and hemoglobin (Hb) are associated with worse survival [216,217,232]. These non-specific parameters are likely to reflect disease activity and systemic inflammation. However, none of these are specific to MPM, and could simply relate to malignancy, chronic disease, or infection.

Several novel circulating biomarkers have been evaluated in an attempt to stratify MPM prognosis. The most extensively studied markers are glycoproteins mesothelin/SMRP, osteopontin, and fibulin-3 as well as megakaryocyte potentiating factor. Some studies have identified prognostic value for all of them, while others have shown no benefit [233–237]. A systematic review published in 2017 identified 45 studies on circulating biomarkers [7]. Due to considerable variation in study design, patient selection and treatment regimen, the authors concluded that none of them should be used for clinical use before a large-scale high-quality prognostic validation.

2.3.6 Radiological prognostic factors

The available imaging techniques, especially CT, usually underestimate the true extent of MPM. Despite inaccuracies in clinical TNM staging, it has been proven to be an independent prognostic marker in several studies [220,221]. The IASLC staging project reported a median OS of 21, 19, 14, and 10 months in MPM patient with stage I, II, III, and IV, respectively [106]. However, several other radiological methods have been proposed to replace or supplement the TNM T-category. CT-derived tumor volume (TV) has been shown to predict survival after multivariate adjustments [238]. A recent publication studied the tumor extension, i.e. number of tumor sites (graded one to three), and found that it predicted survival as well as clinical TNM stage and TV [239]. In addition, maximal tumor thickness calculating from axial CT-scans correlated with survival in surgically treated patients [6]. Also PET-CT has been used to assess prognosis: several studies have found that increased levels of SUVmax are related with a poor prognosis [240,241]. This could be driven by different metabolic activity in histologic subtypes; epithelioid tumors being less metabolically active than non-epithelioid tumors. In addition, two studies combined TV with metabolic information, namely total glycolytic volume (TGV) [242,243], and showed that TGV was superior to clinical TNM stage in predicting survival.

2.3.7 Histological prognostic factors and tumor immunogenity

The major histologic subtypes are the most important prognostic factors recognized by the WHO classifications of tumors of the pleura [184]. A large retrospective analysis including 4207 patients (68% epithelioid, 18% sarcomatoid, 13% biphasic) showed a median OS of 14.4 months in epithelioid, 9.5 months in biphasic and 5.3 months in sarcomatoid subtypes, respectively [25]. Desmoplastic MPM, a rare variant of sarcomatoid, have similar prognosis than pure sarcomatoid subtype [186].

Several prognostic factors have been studied within the epithelioid subtype, since it is the most common subtype and has the best prognosis. The prognostic importance of morphological growth patterns are well recognized tubulopapillary or trabecular (median OS 17.9-24.9 months) tumors have a more favorable prognosis compared to pleomorphic, solid or micropapillary tumors (median OS 8.1-15.8 months) [185,244]. Another study evaluated the prognostic impact of several nuclear features [245]. Multivariate analyses found that mitotic count and nuclear atypia had an independent prognostic impact. Subsequently, three-tier nuclear grading scores were proposed: grade I (median OS 28 months), grade II (median OS 14 months), and grade III (median OS 5 months). These findings have been since validated in a multi-institutional study [246]. They also suggested that adding necrosis to nuclear grade would further stratify the prognosis [246]. In addition, since both the presence of necrosis and a large number of mitotic cells were independent negative prognostic markers, a mitosis-necrosis score was proposed. Other reported markers of bad prognosis are the presence of lymphatic or vascular invasion, atypical mitoses, and the absence of myxoid stroma [245,247].

The role of immune cells within the tumor microenvironment has gained more attention after the emergence of recent immunotherapy agents in various cancers. The tumor microenvironment in MPM is composed of a mixture of stromal, endothelial, and immune cells (such as T-regulatory, granulocytic, and monocytic myeloid-derived suppressor cells and M2-polarized tumor associated macrophages) and is generally highly immunosuppressive [248]. This immunosuppressive state helps the tumor to evade the host immune system. Several studies have identified different distributions of immune cells within the tumor tissue or pleural effusion, which is explained by the ongoing dynamic changes in the immunoenvironment. The prognostic role of expression of programmed death ligand 1 (PD-L1), which inhibits T-cell function via PD-1 and is one of the targets of checkpoint inhibitors, was retrospectively studied in tumor samples from patients enrolled in a clinical phase three trial [249]. Results showed that out of 214 tumor samples 36% were positive for PD-L1 with 65% of them expressing over 50% of PD-L1. PD-L1 staining was a significantly negative prognostic factor in univariate survival analyses; with a cut-off 50%, the median survival was 10.5 months in PD-L1 "high" and 19.3 months in PD-L1 "low" cases. Another study found that patients with PD-L1 positive tumors had a median OS of 4.8 months compared to 16.3 months in those with PD-L1 negative tumors [250]. One study found that tumor microenvironment enriched with T-lymphocytes and macrophages was associated with higher PD-L1 expression on tumor cells and with aggressive histopathological features, leading to worse prognosis [251]. In addition, the prognostic value of semi-quantitative assessment of inflammatory invading cells either in stroma or tumor was assessed in epithelioid MPM [252]. Results showed that chronic inflammation in the stroma, but not in the tumor, was an independent positive predictor of survival. Similarly, an improved outcome was observed in surgically treated patients with higher intra-tumor infiltration by cytotoxic CD8+ T-cells [253]. On the contrary, Salaroglio et al. found no association of survival with CD8+ T-cells but reported that high amount of intra-tumor T-regulatory cells and myeloid-driven suppressor cells were negative prognostic factors [254]. Several larger prospective studies are now ongoing in an attempt to systematically characterize the clinical utility of the immune microenvironment in MPM.

2.4 Treatment of MPM

The most recent treatment guidelines for MPM are the 2018 British Thoracic Society (BTS) and the 2018 American Society of Clinical Oncology (ASCO) guidelines [8,9]. In addition, the new edition of the joint guideline by European Respiratory Society (ERS) and European Society of Thoracic Surgeons (ESTS) was presented in the 29th annual ERS congress [255].

2.4.1 Surgical treatment

Surgical intervention may be needed for diagnosis, staging, and treatment of MPM patients. The aim of surgical treatment is either palliative symptom relief or an intent for local control by complete macroscopical resection, defined as a removal of all grossly visible and palpable tumor (R1 resection) [256]. It is practically impossible to achieve microscopically free resection (R0) margins due to anatomical circumstances. Thus, surgery alone cannot cure MPM, which have led to search for adjuvant therapies after surgery [257]. Surgical options can be divided into palliative partial pleurectomy (partial removal of parietal or visceral pleura for diagnostic or palliative purposes), curative intent pleurectomy/decortication (P/D, parietal and visceral pleurectomy to remove all gross tumor), extended P/D (parietal and visceral pleurectomy with resection of the diaphragm and

pericardium if required), or extrapleural pneumonectomy (EPP, en bloc resection of the parietal pleura, pericardium, diaphragm, lung, and visceral pleura) [258]. Due to variability in the types of operations that are performed, a taskforce report was published in 2019 in order to standardize surgical-based treatments [257]. After surgery, local, ipsilateral hemithorax is the most common site of recurrence, while recurrence in contralateral lung, abdomen, or more distant sites can also occur [259]. In these cases, second line surgical resection can be an option for selected patients but the evidence to support this is lacking.

Radical surgery with curative intent was first introduced by Butchart in the 1970s, and, currently, the two main options are EPP or (extended) P/D [103]. In general, only patients with low tumor burden, epithelioid histology, good PS, absence of extrathoracic and lymph node involvement, and without significant comorbidities are considered candidates for curative intent surgery [260]. Most of the surgical literature is based on uncontrolled case series or registry studies that are prone to both publication and selection bias. In addition, the lack of standardization of adjuvant therapies, usually combined with surgery, makes interpretation of the efficacy of single treatment modality challenging. The reported median survival in the two most radical procedures varies from 14 to 25 months [241]. A meta-analysis including 22 retrospective studies and 2 small early-phase studies compared these two radical surgical techniques and found only modest, non-significant, differences on 2-year survival [258]. However, P/D had an associated 2.5-fold lower perioperatively mortality. Similarly, morbidity is constantly lower and QoL is higher in patients treated with P/D when compared to EPP [261]. The advantages of EPP compared to P/D are a more standardized procedure, simplified use of adjuvant radiation, and a smaller amount of microscopic disease after the operation. The most frequent complications after these radical operations are cardiac arrhythmias, local and systemic infections, venous thrombosis, bronchopleural fistulas, gastrointestinal complications, and acute respiratory distress syndrome (ARDS) [258].

Mesothelioma and Radical Surgery (MARS) trial was the first multicenter randomized controlled trial that was designed to assess if it is possible to randomize patients into EPP or no surgery in a multimodality setting. The initial screening ended with 257 potentially eligible patients, out of which 50 were subsequently randomly assigned to either EPP or no EPP after induction chemotherapy [262]. Even though the trial was not designed and it did not have power to analyze survival differences, it reported a shorter survival in patients with surgery (median OS 14.4 versus 19.5 months; HR 1.90, 95% CI 0.92-3.93, p = 0.082; adjusted HR 2.75, 95% CI 1.21-6.26, p = 0.016). Similarly, median QoL scores were consistently lower in surgically treated patients, although the difference was not significant. The authors concluded that radical surgery in the form of EPP within multimodality treatment offers no benefits and possibly causes harm to patients, and thus it is not feasible to conduct a larger study [263]. However, the study has been heavily criticized for its low sample size, lack of chemotherapy standardization, poor protocol compliance, and higher postoperative mortality and morbidity in EPP patients compared to previous studies [264]. Nonetheless, many centers have shifted from EPP to less arduous lung sparing surgery during the last decade [18].

The lack of good quality trials makes the role, extent, and procedures of radical surgery controversial and different guidelines have different suggestions: the BTS and ERS/ESTS guidelines suggest to only perform curative intent surgery in clinical trials, whereas ASCO guidelines made a strong recommendation for maximal macroscopic resection in highly selected patients, preferably in the context of multimodality treatment performed in centers of excellence [8,9]. To overcome the lack of high-quality surgical evidence, MARS2 randomized clinical trial is now ongoing. They aim to compare extended P/D against no surgical treatment in combination with pemetrexed/cisplatin chemotherapy [265].

2.4.2 Intracavitary therapies

Additional intraoperative loco-regional treatments aim to improve the local effect of surgery. Adding cytotoxic regimen directly to the tumor surface minimizes systemic adverse effects. The first reports using intracavitary chemotherapy are from 1980s in the field of abdominal surgery, while their use was adopted in the MPM in the 1990s [266]. Different intracavitary chemotherapy regimens have been used with an addition of hyperthermia, which has been shown to improve in vitro cytotoxicity with good tolerability [267]. Sugarbaker et al. retrospectively compared the outcomes of patients treated with either EPP or P/D with or without intracavital heated chemotherapy and found significantly better survival in patients who received additional local therapy (35 versus 23 months) [268]. In addition, clinical benefits with low toxicities have been published in early phase studies using local oncolytic virus therapy, antiseptic povidone-iodine, photodynamic therapy, direct cytokine-mediated immunotherapies, gene-mediated cytotoxic immunotherapies, and chimeric antigen receptor T-cell therapy [269–273]. However, more clinical studies are needed before adapting them for clinical use.

2.4.3 Systemic therapies

The standard first line of treatment in surgically unfit MPM patients is a combination of antifolate (pemetrexed/raltitrexed) and platinum (cisplatin/carboplatin) chemotherapy [8]. Its efficacy was proved in a randomized phase III trial where a median OS of 12.1 months was observed in combination chemotherapy compared to 9.3 months OS with single cisplatin treatment [274]. Similarly, they reported improved QoL in the combination therapy arm. A subsequent nonrandomized open-label study, with over 1700 patients, confirmed similar efficacy with pemetrexed-carpolatin than with pemetrexed-cisplatin [275]. The combination of pemetrexed-cisplatin demonstrated a RR of 26.3% compared to 21.7% for the pemetrexed-carboplatin group. Pemetrexed maintenance therapy after 4 to 6 cycles of combination therapy has been studied in a phase II randomized trial of 49 patients [276]. The study reported no difference in PFS (HR 0.99, 95% CI 0.51-1.90) or OS (HR 0.86, 95% CI 0.44-1.71). The addition of anti-VEGF, bevacizumab, to the combination chemotherapy for 4 to 6 cycles followed by bevacizumab maintenance therapy increased the median survival by 2.7 months (HR 0.77, 95% CI 0.62-0.95) at the expense of increased toxicities, treatment cessations, and treatment costs [277]. However, other clinical trials have failed to demonstrate any clinical benefit from anti-angiogenetic drugs, which explains why this druggable pathway have failed to translate into wide general practice [278,279]. In patients with multiple comorbidities or frailty, single agents of pemetrexed, gemcitabine or vinorelbine could be offered even though the response rates are lower than with combination therapy [8,9]. Clinical trials on systemic chemotherapy have not shown big differences in efficacy among histological subtypes and thus similar approaches should be used regardless of the histology.

There is no recommended standard second line therapy for patients who have progressed after platinum-antifolate chemotherapy. Either single-chemotherapy, or retreatment with pemetrexed-based chemotherapy can be used in patients who achieved durable disease control in the first-line setting [8]. The emergence of immune checkpoint inhibitors has revolutionized treatments in many types of cancer, such as NSCLC. In MPM, there have been a several phase I-II studies with ongoing phase III trials. Preliminary results on single immunotherapy agents have shown some antitumor activity, but these results indicate that different combinations are needed to enhance the effects of treatment [280]. For example, the initial observation on pembrolizumab in PD-L1 positive previously treated patients showed 20% RR with 12 months median duration of response, which is comparable to RR of 24% and 7-months median duration of response in patients treated with another anti-PD-1

drug, nivolumab [281,282]. However, these original findings were not confirmed in a multicenter randomized phase III trial on 144 MPM patients, where the efficacy of pembrolizumab was tested against the investigators choice second line chemotherapy [283]. They found improved RR with pembrolizumab (22% versus 6%, p = 0.004) but no differences in PFS (HR 1.06, p = 0.760) or OS in PD-L1 positive (HR 1.47, p = 0.320) or negative (HR 0.72, p = 0.530) patients. Similarly, anti-CTLA4 antibody tremelimumab failed to show improved outcome in second line setting, when tested against placebo in a large (n = 571) multicenter randomized phase II trial (HR for mOS 0.92, 95% CI 0.76-1.12) [284]. In contrast, MAPS2 trial was a multicenter randomized phase II trial comparing PD-1 antibody nivolumab either alone or in combination with CTLA-4 antibody ipilimumab [285]. Both regimens showed anti-tumor activity with 12-week disease control achieved in 44% of cases leading to a median OS of 11.9 months in nivolumab group and 50% and 15.9 months in the combination group, respectively. Similarly, the combination of tremelimumab and durvalumab met phase 2 trial's primary endpoint with 65% disease control rate, leading to PFS of 8.0 months (95% CI 6.7-9.3) and median OS of 16.6 months (95% CI 13.1-20.1) [286]. These studied have showed comparable autoimmune toxicities compared to NSCLC studies. In addition, both objective and prolonged responses were seen in every histological subtype. To date, ERS/ESTS and BTS guidelines suggest waiting for ongoing phase III trials, while some other countries support using these regimen as a second line treatment [9,280].

Among immunotherapies, other novel treatments that have showed positive responses in early phase studies include different cell-based therapies (e.g. targeting microRNAs, dendrite cells or mesothelin), vaccine therapies, diverse tyrosine kinase inhibitors, and arginine deprivation in arginosuccinate synthetase 1-deficient tumors [287–291]. The benefit of the latter has been shown mainly in non-epithelioid patients, which differs from other available therapies. Tumor Treating Fields is a novel portable device that delivers specific electric frequency which interferes with cancer cell proliferation. It has shown promising antiproliferation effect on mesothelioma cells *in vitro* as well as median OS of 18.2 months, when combined with chemotherapy in a single arm phase II study [292]. In addition, comprehensive understanding of the mutational and transcriptomic landscape of MPM has unveiled possible druggable pathways in ongoing clinical trials [293].

2.4.4 Radiation therapy

The rationale for using radiation therapy (RT) in MPM is based on in vitro sensitivity studies and animal models. Radiation therapy has been traditionally used for palliation, prevention for procedure tract metastasis, or as a (neo)adjuvant with surgical resection [294]. The role of high-dose RT has been usually studied as part of a multimodality treatment, which makes the interpretation of its singular benefits difficult. Treating the entire pleura requires a large radiation field, which increases the risks of toxicity [295]. Thus, novel techniques such as intensity-modulated radiation therapy (IMRT) have been studied as it allows higher radiation doses to the tumor while simultaneously sparing normal tissues. Disadvantages include dose inhomogeneity and radiation into the contralateral lung, which can lead to challenging lung complications, especially after EPP when the patient only has one remaining lung [296]. Most severe RT-related adverse effects include radiation pneumonitis, nausea, dyspnea, cough, fatigue, pericarditis, vomiting, esophagus or skin irritation [294,297]. Most of the side effects are mild grade I-II and their incidences is highly depending on dose and treatment regimen. There is no clear consensus on which modalities, dose, or fractionation should be used. Therefore, guidelines on role of RT differ: ERS/ESTS suggest using RT after P/D or EPP only within the context of clinical trials, BTS do not recommend RT other than for pain relief, whereas ASCO suggest using either neoadjuvant or adjuvant RT in the context of multimodality treatment, preferably in centers of excellence [8,9].

A population based study from the Surveillance, Epidemiology, and End Results (SEER) database included 14,228 patients with mesothelioma [298]. They found no survival benefit when they compared outcomes of patients who received surgery alone to those who received it in combination with RT. The role of postoperative RT has been evaluated in one international phase II study, where 151 patients were randomized into either adjuvant RT or no RT after neoadjuvant chemotherapy and EPP [299]. However, due to problems in recruiting and a high drop-out rate, only 23 patients completed the RT-arm as planned. They found no clear benefit from postoperative RT in relapse-free time or OS, but the adverse effects were significantly higher in the RT group. These findings have been criticized due to the many limitations of the trial's design and conduct [300]. In contrast to these two studies, several non-randomized studies have found that postoperative RT is feasible and shows a marked reduction in locoregional recurrence mainly using different techniques [295,297]. For example, Kostron et al. reported that the addition of adjuvant RT after EPP resulted in significantly less loco-regional recurrence (19% versus 47%; $p = 0.003$) [259]. Concurrently, the distant metastases occurred more frequently (36% versus 13%; $p = 0.008$), and there was no significant impact on survival. More recently, a phase III randomized trial compared the outcomes of patients treated with radical hemithoracic RT versus standard palliative RT after lung-sparing surgery and chemotherapy [301]. The long-term results are awaited but preliminary results indicated that more radical RT led to an improved 2-year survival rate of 58% compared to 28% in the standard RT arm, although this occured at the expense of increased toxicity rates.

MPM can spread through previous procedure tracts resulting in painful metastases. One small randomized study consisting of 40 patients found a significant benefit from prophylactic RT, while two more comprehensive and recent randomized phase III trials found no differences in the incidence of procedure tract metastasis [37,302,303]. Moreover, they reported no benefit in QoL, symptom control or survival. Even though the RT doses were small, skin related side effects occurred in approximately half of the patients. In 2018, these results were confirmed in a systemic review and meta-analysis, which included five prospective randomized controlled trials [304]. Thus, prophylactic RT is not generally recommended, while RT is an effective option if a procedure tract metastasis already exists [8,9].

2.4.5 Multimodality treatment

The observation that even the most radical surgery typically results in local recurrence lead to the addition of adjuvant therapies. Multimodality treatment refers to a combination of chemotherapy and RT with radical surgery. Both adjunctive therapies can be used either pre- or postoperatively [18]. A number of non-randomized clinical trials have shown that multimodal treatment is feasible in selected groups of patients. The analysis of 3101 patients from the IASLC database showed superior OS for patients who received multimodality therapy, compared to patients with only curative-intent surgery (20 months versus 11 months) [113]. A systematic review on multimodality treatment including 16 studies was conducted in 2012 [21]. This review included five prospective studies with reported mOS ranged from 14.4 to 25.5 months in the intention-to-treat analysis. When all studies were combined, DFS ranged from 10 to 16.3 months, perioperative mortality from 0 to 12.5%, and morbidity from 50 to 82.6%. The authors concluded that multimodality treatment may offer acceptable perioperative outcomes and long-term survival in selected patients treated in specialized centers. However, due to non-standardized treatment regimens, and the absence of good quality clinical trials, there are uncertainties about the right therapeutic protocol for individual patients.

2.4.6 Palliative treatment

Palliative treatment is designed to relieve symptoms, reduce treatment related side effects, and improve QoL in patients with life-threating diseases and their families. Palliative care is given simultaneously with other cancer treatments from diagnosis to the end of life. In contrast to many other malignancies, a recent randomized study did not find clear benefit of early palliative care implementation in MPM [305].

Dyspnea is the most common symptom in MPM and is mainly due to recurrent pleural effusion. Therapeutic options include repetitive thoracentesis, indwelling pleural catheters, pleurodesis via talc slurry or poudrage, and palliative surgery. There were no differences in patient-reported dyspnea or QoL, when indwelling pleural catheter was tested against talc slurry in a randomized study of 106 malignant pleural effusion patients (approximately 10% of MPM) [306]. Palliative surgery should be considered in eligible and symptomatic patients who do not respond to conventional conservative treatments. One open-label randomized controlled trial has evaluated the role of palliative surgery on symptoms and pleural effusion [307]. It compared palliative partial pleurectomy by VATS to talc pleurodesis. They found no survival benefit with surgery at 12 months but pleural effusion control and QoL improved with surgery at the expense of increased complications, costs, and hospital stay. The authors concluded that talc pleurodesis is the preferred method, but palliative surgery remains an option for certain patients. Moreover, palliative pleurectomy can free entrapped lung with symptomatic improvements [308].

Pain in MPM patients can be due to previous treatments or locoregional tumor invasion into adjacent structures. Palliative RT is an effective option to relieve pain, while it can also be used to resolve hemoptysis, prevent spinal cord compromise, and improve cough [294]. In pain relief, most of the evidence comes from retrospective series, while two small prospective trials have been conducted with reported pain relief in 47% and 68% of patients with minimal improvements in QoL but no significant side effects [309,310]. The prospective studies reported no significant differences between histological subtypes in terms of response. The recommended palliative dosing regimen for pain relief depends on the affected tissue, and can include 8 Gy single dose, 4 Gy five times or 3 Gy 10 times [8]. Opioids are the most common drugs that are used in cancer related pain. They may also deviate dyspnea, cough and anxiety, which makes them useful in MPM symptom management. Local anesthetics, such as lidocaine, can be used in direct chest wall pain. Epidural or intrathecal treatments can be used in refractory cases [311]. Other possible drugs to alleviate symptoms are anti-convulsants, corticosteroids, tricyclic antidepressants, and benzodiazepines [311].

3. Aims and hypothesis

Previous publications have suggested that CT-based tumor volume assessment could provide accurate tumor burden evaluation with prognostic implications [5]. Moreover, unidimensional measurements of tumor thickness have been universally used for tumor response estimations [90]. Thus, we hypothesized that measuring tumor thickness with or without its circular and vertical pleural extent in CT could reflect tumor size and its association with survival in our cohort. Secondly, activin A has been proven to be overexpressed in MPM tissue and cells in studies in our institution [10]. We hypothesized that circulating levels of activins could be elevated in MPM patients and that preclinical associations could be confirmed in a clinical setting. Thirdly, we hypothesized that cancer cachexia, occurring frequently in other thoracic malignancies, is a prevalent condition also in MPM. In addition, weight loss, a hallmark of cachexia, is a common finding also in MPM [312]. Fourthly, since the diagnosis of MPM can be difficult and the overall prognosis is dismal, we hypothesized that a proportion of patients with prolonged survival are wrongly diagnosed at baseline, and we re-evaluated these patients who formed a long-term survivor cohort.

The aims of this doctoral study were to evaluate clinical, radiological and histopathological factors that associate with the overall survival in MPM. The specific aims in studies I to IV were:

I) To define a simple and accurate method to approximately determine radiological tumor size and test its prognostic utility compared to other radiological characteristics (study I)

II) To prospectively analyze the role of circulating activins and follistatins in MPM (study II)

III) To evaluate the prevalence of cancer cachexia in MPM (study II)

IV) To confirm the diagnosis of MPM patients with extended survival, and search for histopathological (study III) and clinical (study IV) factors that predict prolonged survival.

4. Materials and methods

4.1 Study population

All MPM patients diagnosed in Finland between 2002 to 2012 were identified from the FCR. These patients conformed the population base for studies I, III, and IV. Patients from the Helsinki University Hospital (HUS) region with available diagnostic CT scans formed the population of study I. From these patients, we selected the ones having epithelioid histology with survival less than five years to establish a control group for studies III and IV. The epithelioid subtype was chosen as a control group because of the assumption that the long-term survivals (LTS) would be of epithelioid subtype. After thorough histopathological and clinical confirmation of MPM diagnosis, all MPM patients with OS over five years were characterized as LTS in studies III and IV. The follow-up for study I closed on February 2017, for study III September 2018, and IV in January 2018.

For study II, we prospectively enrolled patients with a suspected thoracic malignancy from June 2016 to January 2018 in the HUS area. The study was conducted as a pilot study for the newly established Helsinki Biobank; hence recruited patients provided a written Biobank consent. We recruited patients before either diagnostic- or therapeutic surgery. This approach was decided upon to ensure that good quality tissue samples were available for firm MPM diagnosis. Patients with blood samples obtained postoperatively and an uncertain diagnosis or rare diagnosis were excluded from the study.

Figure 4 shows a detailed flowchart of the populations in studies I to IV.

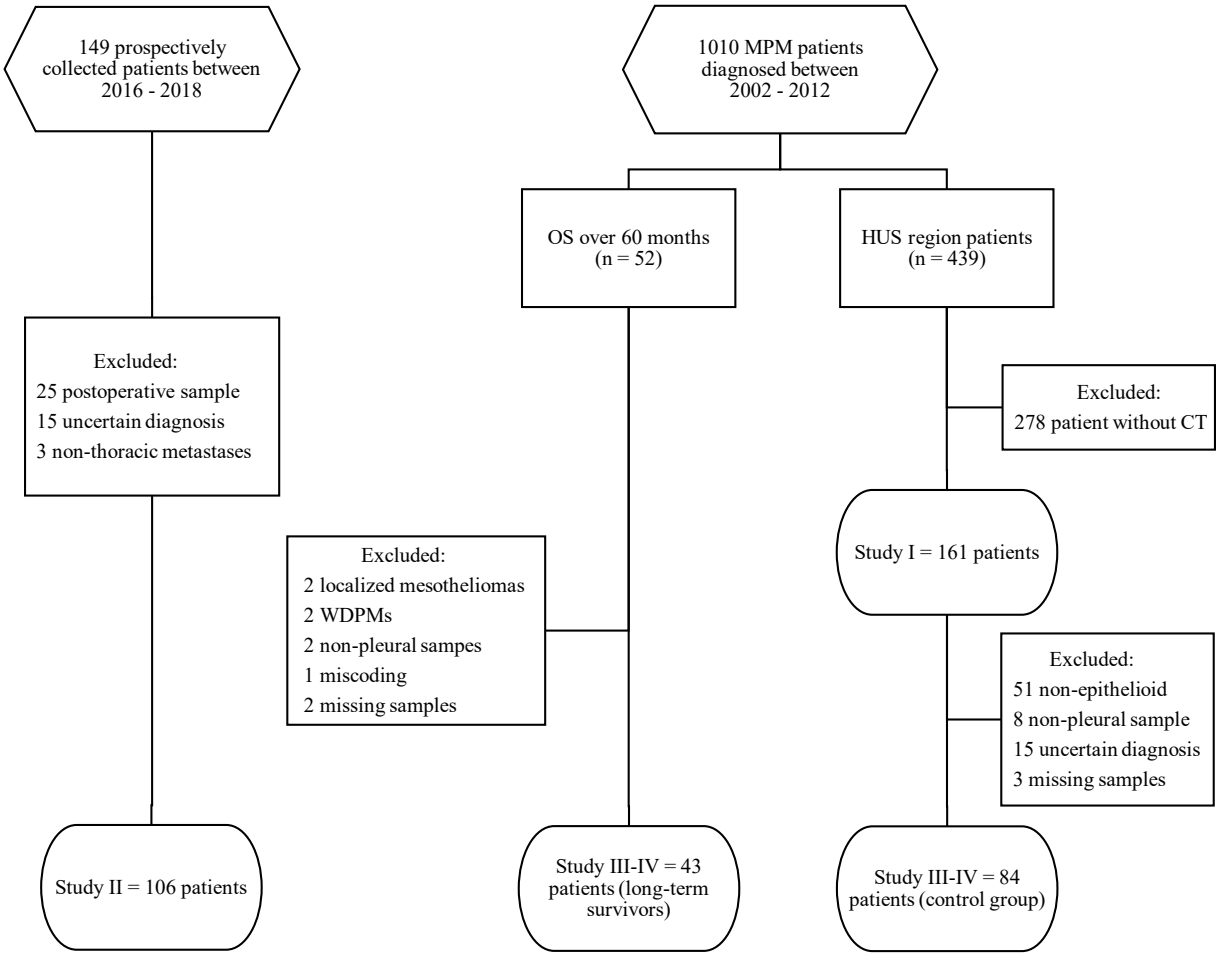


Figure 4. Flowchart of patients in studies I to IV. MPM, malignant pleural mesothelioma; OS, overall survival; HUS, Helsinki University Hospital; CT, computed tomography; WDPM, well-differentiated papillary mesothelioma

4.2 Methods

4.2.1 Patient and disease information

Finnish Cancer Registry has maintained a nationwide cancer registry since 1953. FCR data are based on information collected from clinicians and pathologists [1]. For studies I, III, and IV the information from the FCR was complemented by mortality data from the National Registry of Causes of Death at Statistics Finland. In addition, individual clinical data used in all of these studies were collected from hospital medical records. This clinical information was transferred and stored into a secure, certified electronic database maintained by Granitics Unify Med.

In addition to the basic patient characteristics, multiple categorizations and computational measures were used in these studies. Performance status was collected or estimated (if not readily available) from the hospital medical records using WHO/Eastern Cooperative Oncology Group (ECOG) [313]. Comorbidities were measured using Charlson comorbidity index (CCI) score [314]. Smoking status was dichotomized into either never-smokers or former/current smokers with calculated pack-years. Body mass index (BMI) was calculated by dividing weight by the square of height and expressed as kg/m^2. Patients were defined as cachectic in study II, if they presented with over five percent weight loss over the past six months, if they had BMI below 20 kg/m^2 with weight loss of over two percent, or CT-based skeletal muscle index (SMI) consistent with sarcopenia and weight loss of over two percent [167].

In these studies, a set of different approaches was used to obtain information about prior asbestos exposure. Decisions regarding worker's compensation benefit were gathered from the National Workers' Compensation Center Registry. Pleural plaques and calcifications are markers of prior asbestos exposure, and their presence were quantified from CT scans. Possible occupational asbestos exposure was documented from medical records. In addition, if available, pulmonary asbestos fiber counts were measured from autopsy lung samples using scanning transmission electron microscopy (STEM) at the Finnish Institute of Occupational Health.

4.2.2 Computed tomography assessment

Study I was based on CT characteristics. Also, for studies II and IV pretreatment CT scans were evaluated at the time of diagnosis. If available, contrast-enhancement chest and upper abdomen CT scans were preferred over other CT modalities. In all studies, CT images were measured blinded to the clinical information by a single radiologist. In study II, additional post-chemotherapy CT scans were evaluated to assess response to chemotherapy. The assessment of response was done by using both the mRECIST criteria (version 1.0) and the change of tumor size (TS) estimation [90].

The CT-based clinical staging was utilized using 8^{th} edition of the AJCC/UICC TNM classification. Because of the small number of patients in different stage groups, we combined stage groups IA, IB with II and IIIA with IIIB in study I, and IA with IB and IIIA with IIIB in study II, IV.

TS estimation was used in studies I, II, and IV. First, the maximal tumor thickness was evaluated in axial planes perpendicular to the chest wall or mediastinum. Second, to approximate the vertical and circular extension of the tumor, we divided the pleural cavity into three zones. For each of the three zones, the slice with the greatest circular extension of the tumor was evaluated separately. The final extension was summated at the level of the carina, and a four-tier scale was used: 0 = no tumor, 1 ≤ 90º, 2 = 90-180º, 3≥180º (Figure 5) [315]. For example, if a small (less than 90º) tumor is located

only in the upper zone, the extension grade would be 1. If a similar tumor extension would be found also in the middle and lower zone, the grade would be 2 or 3 depending on the final summation. Finally, the TS was estimated by multiplying the extent grade with the maximal tumor thickness.

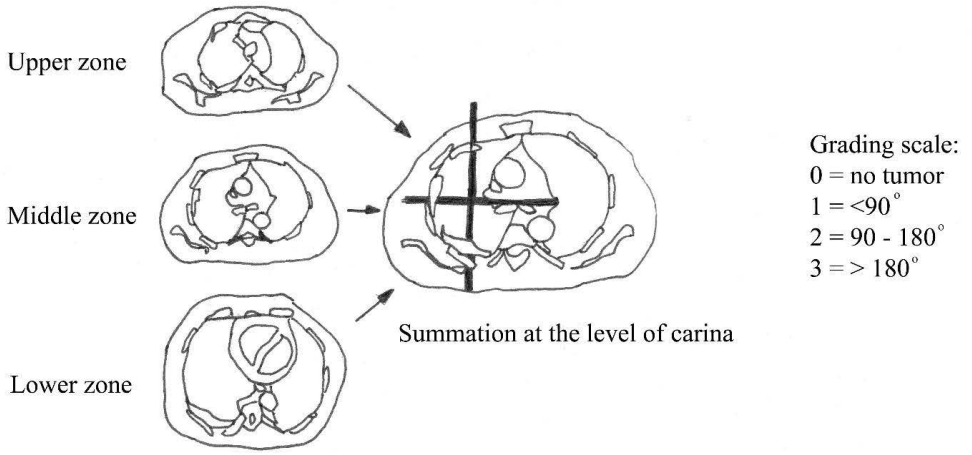


Figure 5. The tumor extension grading scale in the pleural cavity [315].

In study I, pleural effusion was measured using its maximal axial thickness. Fibrosis and emphysema scores were evaluated on a previously published scale, where 0 represents normal findings and 5 extreme changes [316,317]. A set of 30 (19%) images were randomly re-evaluated after a month by the same radiologist to determine the intra-rater agreement.

For study II, individual SMI used in cachexia assessment was evaluated from pretreatment CT images according to a previously published method [318]. The cross-sectional areas of the psoas, quadratus lumborum, paraspinal, transverse abdominal, external oblique, internal oblique, and rectus abdominis muscles were identified at the level of vertebra L3. These muscle areas were quantified using Osirix© version 33 (32-bit Pixmeo, Sarl, Switzerland). SMI was calculated correcting the skeletal muscle area for height (m^2). The cut-off used for defining sarcopenia was based on previous consensus report: SMI below 55 cm^2/m^2 for men and 39 cm^2/m^2 for female [167].

4.2.3 Activin assays and other laboratory measurements

For study II, pre-treatment serum samples were prospectively collected from participating patients. The serum was stored in the Helsinki Biobank after centrifugation at -80˚C. Serum activin A, activin B, FS, and FSTL3 levels were measured by ELISA from AnshLabs LLC, Webster, USA. A BioRad Benchmark microplate reader was used for activins measurements and Hidex Sense for FS and FSTL3 measurements. The absorbance was measured at 450 nm to quantify biomarker levels. In addition, CRP levels (cutoff of 3 mg/L) and hemoglobin were measured at the time of the biomarker collection as part of the routine preoperative workup and included in the study.

4.2.4 Histopathological evaluation

Studies III and IV were based on histopathological confirmation of patients with a survival of over five years. First, the initial diagnoses were confirmed, along with control group, by evaluating tumor samples blindly to the survival data. Information on clinical and radiological characteristics was added to the histopathological evaluation, if needed. In general, the first diagnostic sample was evaluated but more representative samples were used instead, if it was obtained within three months. If the sample included several tissue blocks, all of these were studied but only one slide was selected for further analyses. The diagnosis was based on the current standards of morphological and IHC criteria [17]. Additional IHC stains were carried out if at least two positive and two negative mesothelial markers were not available on the diagnostic samples. In addition, if enough tumor tissue was available, we constructed formalin-fixed paraffin embedded tissue microarray (TMA) blocks, on which *BAP1* IHC-staining was performed. Figure 2 shows the distribution of excluded patients in these studies.

After the initial diagnosis was confirmed, a second evaluation round was performed blindly to the clinical information in study III. During the evaluation, all samples were classified according to their size into either large, small, or scant, independently of the biopsy method used to obtain the sample. The three-tier nuclear grade was established using a previously published method by adding nuclear atypia and mitotic count [245]. Nuclear size and irregularity were assessed at x 400 magnification, and scored into either mild, moderate or severe atypia. The mitotic count was calculated from the spots with highest mitotic activity using 50 high power field (HPF) and determined the average per 10 HPF. After that, tumors were divided into three-point mitotic score. The final nuclear grade was calculated by adding mitotic score to nuclear atypia scale. In addition to nuclear grade, morphological subtypes were recorded as five percent increments, and classified by predominant growth pattern: trabecular, tubulopapillary, solid, micropapillary, or pleomorphic. For survival analyses, these subgroups were classified into low-grade (trabecular, tubulopapillary) or high-grade (solid, micropapillary, pleomorphic) according to previous publications [185]. We also reported the presence of necrosis, myxoid stroma (positive if it contains over 50% of the tumor volume). In addition to these previously published features, we noted tumor density (the percentage of tumor in the sample evaluated in 10x field), presence of tubular structures covered with a single layer of mesothelial cells (single layer), and the presence of exophytic growth (large stout fibrovascular papillae - “polypoid-pattern”).

4.3 Ethical considerations

All studies were conducted in accordance with the Declaration of Helsinki. Studies I, III, and IV were retrospective observational studies, which includes information from several nationwide registries. Permissions to use these data and tissue samples were received from HUS (HUS/746/2018), Finnish National Institute for Health and Welfare, National Workers Compensation Center and Statistics Finland. Valvira, a national supervisor of the social and health care, has granted permission to study tissue samples (752/06.01.03.01/2016). A statement of approval for the study protocol was given by the Ethical Committee of HUS (418/13/03/02/2015).

Study II was a prospective observational study, that was conducted as a pilot study for Helsinki Biobank. All recruited patients provided a written Biobank consent. Research permission was granted by the Institutional Review Board of HUS (HUS/152/2016), Scientific Steering Committee of the Helsinki Biobank (HUS/359/2017), and the Ethical Committee of HUS (HUS/1057/2019).

4.4 Statistical methods

In all studies, categorical variables are presented as number of patients with percentages, and continuous values as medians with range or interquartile range (IQR) or mean with standard deviation (SD). All reported p-values are based on two-tailed tests. Bonferroni correction for multiple tests was used in studies II and III, if multiple comparisons were made. The chi-square test was used for testing between categorical variables and Mann-Whitney's U-test or Kruskal-Wallis test for continuous variables. The nonparametric Spearman correlation coefficient was used to assess the relationships between two continuous variables. Intra-observer agreement was defined using weighted kappa ($w\kappa$) for categorical variables and intra-class correlation (ICC) for continuous variables in study I.

The survival analyses were carried from the date of diagnosis to the end of follow-up or until death. Both cancer-specific and all-cause mortality were used in the analyses. Since the vast majority of patients died of MPM, no significant differences were noted between these mortality types. Survival curves were computed using the Kaplan-Meier method, and the statistical significance was determined using the log rank test. The Cox's proportional hazards regression model was used to estimate the strength of the association between various factors and survival.

In study I, the predictors for survival were first identified by univariate analyses. The significant predictors found were then included into a multivariate model consisting of CT-based TS evaluation, laterality, pleural effusion, TNM stage, pleural calcification, along with histological subtype, sex, and age. Histopathological factors that had shown association with survival before or identified in univariate analyses (nuclear grade, histologic subtypes, necrosis, exophytic polypoid growth, single layer, and tumor density) were entered into a multivariate model in study III. Similarly, the final multivariate model in study IV consisted TS, clinical TNM stage, occupational disease, and ECOG PS. In addition, both models were adjusted with age, sex, and treatment status. Study II focused on associations between clinical endpoints and circulating biomarker levels. The logistic or linear regression analysis was used to determine the independent effect of activin A on cachexia or chemotherapy response, using adjustments for age, sex, CRP, and TS.

Statistical analyses in Study I were performed using SAS System for Windows version 9.4 (SAS institute, Inc. Cary, NC) or SPSS version 24.0 (IBM SPSS Statistics, Chigaco, IL), and in study II-IV using SPSS version 25.0. In all studies, a p-value less than 0.05 was considered significant.

5. Results

5.1 Patient baseline characteristics

Table 5 presents the characteristics and distributions of MPM patients included in our studies. In summary, we found that most of MPM patients were old men with previous asbestos exposure. Apart from the LTS group, the median survival was scarce, and most patients died due to MPM. In study II, biomarker levels were compared to NSCLC and benign lung tumor patients. The distributions of baseline information between main study groups can be viewed in the study II attached at the end of the thesis.

Table 5. Malignant pleural mesothelioma patient demographics in studies I to IV

Characteristics	**Study I**	**Study II**	**Study III-IV**	
			LTS[1]	**Control**
Patients	161	21	43	84
Age, years, (median, range)	69 (43-89)	71 (63-77)	61 (27-87)	67 (54-84)
Sex, male, n (%)	138 (86)	20 (95)	30 (70)	74 (88)
Asbestos exposure, yes, n (%)	109 (68)	NA[2]	18 (42)	63 (75)
CCI[3], mean (SD)[4]	NA	0.8 ± 1.2	0.60 ± 0.8	0.67 ± 0.9
ECOG PS[5], n (%)				
0	NA	13 (62)	39 (91)	33 (39)
> 1		8 (38)	4 (9)	51 (61)
Histology, n (%)				
Epithelioid	111 (69)	14 (67)	45 (100)	84 (100)
Biphasic	23 (14)	3 (14)		
Sarcomatoid	27 (17)	4 (19)		
Stage[6], n (%)			*	
No visible tumor	12 (7)		7 (18)	4 (5)
Stage I	46 (29)	3 (14)	16 (41)	27 (32)
Stage II	5 (3)	2 (10)	3 (8)	3 (4)
Stage III	74 (46)	14 (66)	11 (28)	37 (44)
Stage IV	24 (15)	2 (10)	2 (5)	13 (15)
Tumor size, mm, median (IQR)[7]	36.0 (12.0-78.0)	45.0 (2.90-61.5)	17.0 (2.3-45.3)	30.0 (10-62.3)
OS[8], median (IQR)	9.1 (3.3-19.7)	7.6 (4.5-11.8)	79.3 (69.3-99.3)	11.3 (5.6-19.2)
Cause of death, n (%)				
MPM[9]	153 (95)	8 (38)	32 (74)	83 (99)
Other cause	2 (1)	1 (5)	3 (7)	1 (1)
Alive	6 (4)	12 (57)	8 (19)	0

* data missing from 4 patients; 1. LTS, long-term survivals; 2. NA, not available; 3. CCI, Charles comorbidity index; 4. SD, standard deviation; 5. ECOG PS, eastern cooperative oncology group; 6. refers to clinical stage; 7. IQR, interquartile range; 8. OS, overall survival; 9, MPM, malignant mesothelioma

5.2 Computed tomography and mortality (study I)

There were 161 MPM patients at HUS district with diagnostic CT-images available. An overview of the most common CT characteristics is found in Table 6. The most common CT finding was unilateral pleural thickening with pleural effusion. Some form of emphysema was noted in 50 (31%) and fibrosis in 41 (26%) of patients. Most patients had a stage III disease, while 12 (7%) patients had no recognizable tumor in the CT scan (Table 5).

Table 6. Overview of radiological characteristics (n = 161)

CT finding	**Value**
Pleural thickening, n (%)	149 (93)
Pleural effusion, n (%)	144 (89)
Pleural effusion (mm); median (range)	50.0 (0-165)
Tumor thickness (mm); median (range)	18.0 (0-102)
Tumor extent grade; n (%)	
Grade 0	12 (7)
Grade 1	53 (33)
Grade 2	32 (20)
Grade 3	64 (40)
Tumor size (mm); median (range)	36 (0-306)
Pleural plaques, n (%)	115 (71)
Pleural calcification, n (%)	98 (61)

The estimation of TS associated with different MPM histological subgroups. Largest size was noted in sarcomatoid subtype (median 66.0, $p = 0.003$). The TS increased along with advanced tumor stage ($p < 0.001$), as well as correlated with TNM T-class ($r = 0.70$, $p < 0.001$) and N-class ($r = 0.41$, $p < 0.001$). The intra-observer agreement for TS estimation was excellent (ICC 0.93, 95% CI 0.86-0.96). Other measurements can be found in the original publication (study I) at the end of this thesis.

The median survival-time was 9.1 (range 0-104) months. Univariate survival analyses showed that age ($p = 0.061$), laterality ($p = 0.067$), TS ($p < 0.001$), the amount of pleural effusion ($p = 0.038$), the presence of bilateral pleural calcification ($p = 0.046$), stage ($p = 0.07$), and histological subtype ($p < 0.001$) were associated with mortality. In multivariate analyses, high TS (HR 1.01, 95% CI 1.01-1.02, $p < 0.001$) and amount of pleural effusion (HR 1.01, 95% CI 1.01-1.02, $p < 0.001$) along with sarcomatoid histology (HR 4.71, 95% CI 2.88-7.69, $p < 0.001$) were independent predictors of poor survival. When TS was divided into its initial factors, both tumor thickness, and pleural extent grade predicted survival in univariate analyses, but only extent grade was significant ($p = 0.001$) after adjustments. The Kaplan-Meier survival plot is shown in Figure 6, when TS was dichotomized by its median.

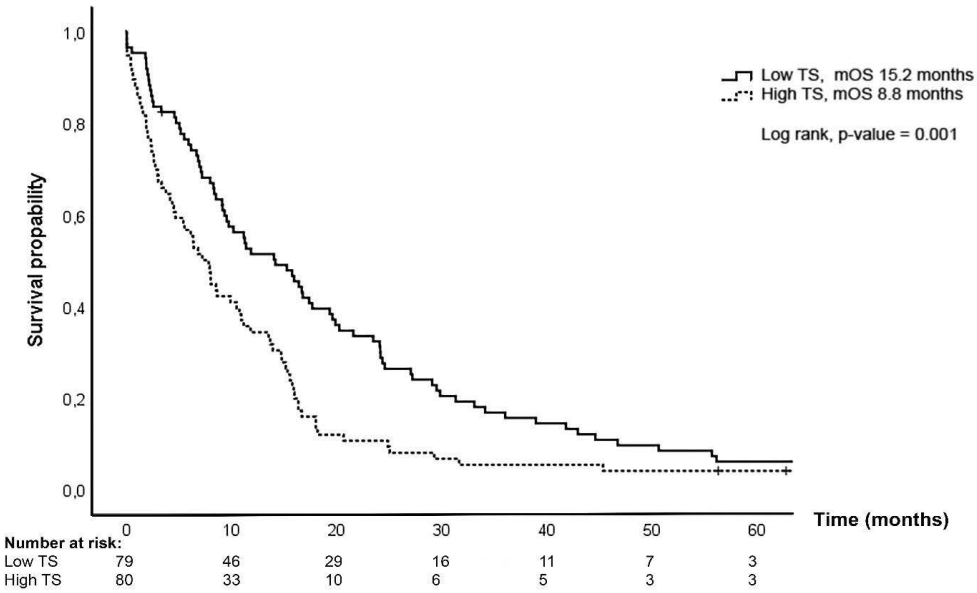


Figure 6. Kaplan-Meier curve showing survival according to tumor size. TS, tumor size, mm; mOS, median overall survival

5.3 Circulating activins and follistatins in MPM (study II)

A total of 106 patients were recruited in study II. The main study groups consisted of 21 (21%) MPM, 59 (58%) NSCLC, and 22 (21%) patients with benign lung tumors. Table 7 summarizes the values of different circulative biomarker levels in these groups. All biomarker levels were the highest in MPM. Follistatin and FSTL3 levels showed significant differences across the treatment groups, whereas circulating activin A was the best to discriminate between these groups. None of the biomarkers differentiated between histological subtypes of MPM or NSCLC.

Table 7. Circulating biomarker levels in the main study groups. The P-values indicates the differences between these groups.

	MPM[1] (n=21)	NSCLC[2] (n = 59)	Benign tumor (n=22)	P-value
Activin A, median (IQR)[3], ng/ml	1.21 (0.90-2.02)	0.68 (0.49-0.85)	0.40 (0.32-0.54)	<0.001
Activin B, median (IQR), ng/ml	0.12 (0.10-0.18)	0.10 (0.09-0.13)	0.10 (0.08-0.12)	0.121
Follistatin, median (IQR), ng/ml	3.50 (2.48-3.98)	2.51 (1.93-3.06)	2.34 (1.77-2.99)	0.013
FSTL3, median (IQR), ng/ml	14.75 (11.13-35.13)	11.25 (10.00-14.50)	9.13 (7.31-10.06)	<0.001

1. MPM, malignant pleural mesothelioma; 2. NSCLC, non-small cell lung cancer; 3. IQR, interquartile range

Twelve (57%) MPM patients were cachectic compared to seven (12%) NSCLC patients at diagnosis ($p < 0.001$). Figure 7 shows different biomarker levels according to cachexia: activin A was the only marker that was statistically higher in patients with cachexia ($p = 0.001$). Circulating activin A levels correlated also with age ($r = 0.414$; $p < 0.01$), and CRP ($r = 0.468$; $p < 0.01$). The association with cachexia and activin A remained significant after adjustments for age, sex, CRP ($p = 0.047$).

In MPM patients, activin A was the only biomarker that was positively correlated with pretreatment CT-based TS assessment ($r = 0.549$; $p = 0.010$) but did not associate with TNM stage ($p = 0.542$). Twelve (57%) MPM patients received first-line platinum-based chemotherapy. When comparing TS change between pre- and post-chemotherapy, we found that patients with progressive disease had almost three-fold higher circulating activin A levels compared to patients with partial response or stable disease (median 3.09 versus 1.21 ng/ml; $p = 0.0028$). This association remained significant after adjustments for TS, CRP, sex, and age ($p = 0.008$). The findings were similar when assessing the chemotherapy response with mRECIST criteria, and mRECIST correlated positively with TS change ($r = 0.918$, $p < 0.001$).

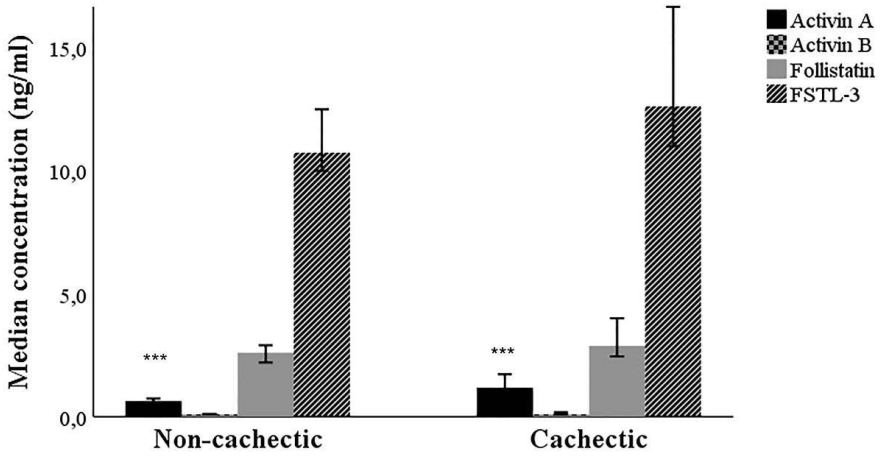


Figure 7. Median levels of activins and follistatins in patients with cancer cachexia. Activin A levels were higher in cachectic patients (*** indicates significant p-value).

5.4 Clinical and histopathological factors and long-term survival (study III-IV)

Figure 2 summarizes the diagnostic validation process for both LTS and control group. A total of 127 tumor specimens were analyzed: 43 (34%) were LTS and 84 (66%) formed the control group. A median of three (IQR 1-5) H&E slides were reviewed from each tumor. The diagnostic accuracy of the original MPM diagnosis was good as only one (0.5%) NSCLC patient was miscoded at the cancer registry as MPM. In addition, we identified two LMMs (0.9%) and two WDMPs (0.9%) that were originally diagnosed as diffuse MPM. *BAP1* IHC was available in 57 (45%) patients and was negative in 12/14 (86%) LTS patients and 25/43 (58%) control patients ($p = 0.150$).

Clinical characteristics can be viewed in Table 5. Compared with the control group, the LTS were younger, more frequently females, and their performance status was better at the time of diagnosis. The proportion of prior malignancies was similar in both study groups ($p = 0.213$). LTS patients were more likely to undergo surgery with a p-value trending towards significance (44% versus 27%; $p = 0.057$). LTS patients were more likely to be treated with chemotherapy ($p = 0.048$) or RT ($p = 0.014$).

The main histopathological differences between the study groups are summarized in Table 8. In summary, LTS tumors had mostly tubulopapillary growth pattern with nuclear grade I, whereas solid growth pattern and grade II were more common in controls ($p < 0.001$). All but one tumor had exophytic polypoid growth pattern in LTS tumors ($p < 0.001$). In contrast, one LTS tumor had necrosis compared to sixteen in control group ($p <0.007$).

Table 8. Main histopathological findings according to study groups

	LTS[1]	Control	p-value
Sample size, n (%)			0.123
Large	33 (77)	52 (62)	
Small	7 (16)	28 (33)	
Scant	3 (7)	4 (5)	
Nuclear grade, n (%)			<0.001
Grade I	34 (90)	28 (34)	
Grade II	4 (10)	49 (61)	
Grade III	0	4 (5)	
Histologic subtypes, n (%)			<0.001
Trabecular	2 (5)	0	
Tubulopapillary	30 (70)	24 (29)	
Solid	9 (20)	59 (70)	
Micropapillary	2 (5)	1 (1)	
Compound histologic subtype[2], n (%)			<0.001
High-grade	11 (25)	60 (71)	
Low-grade	32 (75)	24 (29)	
Myxoid stroma, n (%)			0.605
Absent	40 (93)	80 (95)	
Present	3 (7)	4 (5)	
Necrosis, n (%)			0.007
Absent	42 (98)	68 (81)	
Present	1 (2)	16 (19)	
Exophytic polypoid growth pattern, n (%)			<0.001
Absent	32 (74)	83 (99)	
Present	11 (26)	1 (1)	
Single layer, n (%)			<0.001
Absent	16 (37)	61 (73)	
Present	27 (63)	23 (27)	
Tumor density, %, median (IQR)	50.0 (30 – 60)	70.0 (45 – 81)	<0.001

1. LTS, long-term survival over five years; 2. Histologic subtypes combined for survival analyses: high-grade (solid, micropapillary), low-grade (trabecular, tubulopapillary)

In study IV, we observed that LTS patients had lower asbestos exposure than control group when measured either by self-reported (44% versus 76%; $p < 0.001$) or occupational compensations (42% versus 75%; $p < 0.001$) as well as lower fiber count concentrations in the lung tissue (median 0.4 mf/g, IQR 0.2-5.4 versus 9.0, IQR 0.6-132.5; $p = 0.019$). In study III, we investigated the association between the fiber count and histopathological parameters. We found that the total concentration of lung fibers associated with nuclear grade, so that the lowest concentrations were found on grade I (median 0.60, IQR 0.20-8.60 mf/g) and the highest in grade III (median 280.0, IQR 150-280 mf/g) ($p < 0.001$). Likewise, higher tissue fiber content was more likely to be found in tumors with necrosis than in tumors without necrosis ($p = 0.027$).

Survival times and cause of death can be seen in Table 5. In study III, univariate survival analyses showed that the histopathological findings that affected survival were nuclear grade ($p < 0.001$), epithelioid histologic subtypes ($p < 0.001$), necrosis ($p = 0.002$), tumor density ($p < 0.001$) and the presence of exophytic polypoid growth ($p = 0.002$) or single layer ($p <0.001$). Similarly, age ($p = 0.002$), TS ($p = 0.002$), ECOG PS ($p < 0.001$), and occupational asbestos disease ($p = 0.008$)

associated with survival in study IV. These variables were entered into two separate multivariate models along with age, sex, and treatment status. The final multivariate models are presented in Table 9. In summary, nuclear grade, ECOG performance status, TS, and the presence of exophytic polypoid growth were independent predictors of survival. When treatment was added to the multivariate model B it showed that both surgery (HR 0.38, 95% CI 0.21-0.69) and chemotherapy (HR 0.48, 95% CI 0.28-0.81) were independent predictors for better survival when compared to patients with best supportive care.

Table 9. Multivariate analysis associated with overall survival

	Study III[A]		**Study IV[B]**	
Variable	**HR[1] (95% CI[2])**	**p-value**	**HR (95% CI)**	**p-value**
Age (continuous)	1.01 (0.99 – 1.03)	0.674	1.02 (0.99 – 1.04)	0.179
Sex, male	1.25 (0.71 – 2.18)	0.440	1.06 (0.57 – 1.96)	0.861
Nuclear grade		<0.001		
Grade I	1.00			
Grade II	4.43 (2.51– 7.82)			
Grade III	16.39 (4.57 – 58.76)			
Histologic subtypes, low-grade	0.71 (0.39 – 1.26)	0.241		
Necrosis, yes	1.26 (0.66 – 2.42)	0.484		
Exophytic polypoid growth, yes	0.43 (0.21 – 0.89)	0.024		
Single layer, yes	0.76 (0.46 – 1.26)	0.288		
Tumor density (continuous)	1.00 (0.99 – 1.01)	0.883		
EGOC[3] performance status				<0.001
PS 0			1.00	
PS I-III			2.97 (1.82 – 4.82)	
TS (continuous)			1.01 (1.00 – 1.01)	0.022
Clinical stage				0.903
No measurable disease			1.00	
Stage I			1.16 (0.55 – 2.45)	0.694
Stage II			1.76 (0.56 – 5.57)	0.334
Stage III			1.27 (0.57 – 2.81)	0.562
Stage IV			1.27 (0.48 – 3.36)	0.631
Occupational disease, yes			1.29 (0.78 – 2.16)	0.314

Histopathological (A) cox proportional hazard model was adjusted for age, sex, nuclear grade, histologic subtypes, necrosis, exophytic polypoid growth, single layer, tumor density, and treatment; Clinical (B) model adjusted for age, sex, ECOG performance status, TS, clinical TNM stage, occupational disease, and treatment; 1. HR, hazard ratio; 2. CI, confidence interval; 3. ECOG, eastern cooperative oncology groups; 4. TS, tumor size, mm.

6. Discussion

This doctoral thesis studied clinical, radiological, and histological factors in MPM and their relation to survival as well as other important clinical endpoints. In summary, we could confirm several previously published prognostic factors as well as observed novel features that may be helpful in predicting survival. The independent radiological prognostic factors found in study I were TS and the amount of pleural effusion among with the histological subgroups. Similarly, when studying only patients with epithelioid subtype in study III-IV TS and ECOG performance status as well as histopathological nuclear grade and the presence of exophytic polypoid growth were the strongest predictors of survival. In addition, we confirmed that circulating activin A is elevated in MPM and is associated with cancer cachexia, TS, and chemotherapy response.

6.1 Patient related prognostic factors

Age, sex, comorbidities and performance status are examples of host-related prognostic factors [207]. Several studies have found their significance in numerous malignancies, including MPM [20,23,221]. The male predominance, observed in our studies is mostly explained by the occupational nature of the disease [35]. Several studies have also observed that male sex is an adverse prognostic factor [23,211]. In study IV, we found that LTS patients were younger and more commonly female than controls, but neither of them affected survival after adjustments. When comparing the baseline characteristics against NSCLC patients in study II, we observed that MPM patients had less smoking history and comorbidities than lung cancer patients. This is not surprising, since smoking is the main etiological factor in lung cancer and also contributes to several other diseases, while no clear associations have been reported between smoking and MPM [2,319].

Contrary to previous results, we did not find any survival associations with comorbidities, when assessed by the Charlson comorbidity scale [221]. Performance status, as evaluated by ECOG scale or other quantitative measurements, is an important and universal measurement in cancer patients [320]. It does not only play a role in prognostic assessment, but also guides treatment options in individual patients. Patients with worse PS tend not to tolerate most of the treatments and are not usually included in the clinical trials. Hence, in general, the prognostic utility of PS cannot be studied in clinical trials, while large population-based studies typically lack this information [211]. Unsurprisingly, in study IV we found that most of LTS patients were PS scale 0 and that PS was also an independent prognostic factor after adjustments. This is in line with previous studies on MPM as well as other cancers [20,320]. The weakness of this evaluation is that it was determined at baseline, and it can substantially change in MPM patients after certain treatments such as thoracentesis. In addition, it is a subjective measurement and had to be estimated afterwards for some patients due to missing information in the medical records.

The role of asbestos as an etiological factor of MPM is firmly established, but its prognostic significance has been debated [211,321]. Some studies have reported a worse outcome in patients with a prior asbestos exposure [222], while others have found no survival associations [211]. Establishing accurate anamnesis for asbestos exposure can be complicated due to a long latency period between asbestos exposure and the MPM development. Moreover, histories and the amount of exposure are generally reliable at a cohort level, but their reliability decreases at the individual level [57]. Non- or para-occupational exposure is even harder to quantify. To overcome these limitations, specific questionnaires and calculations have been developed to capture exposure more

reliably, but these can only be reliably used in a prospective design. Due to these limitations, we measured the role of asbestos in multiple different ways. In study IV, they all showed similar results: LTS patients were less likely to have occupational asbestos disease, they had fewer pleural plaques in CT, and lower asbestos fiber burden in TEM analyses when compared to control group. However, occupational asbestos disease was a significant predictor of survival only in univariate analyses but not after adjustments. Also, the number of total fiber analyses made in LTS patients (31%) was low, which prevented us from reliably studying its prognostic significance or the associations to asbestos fiber types.

In study IV we studied the role of therapy. In line with previous retrospective studies, we found that patients undergoing surgery or chemotherapy were associated with longer survival compared to patients with no therapy [19,222]. This is not surprising, since usually only suitable patients are selected for certain therapies and not all of the confounders can be adjusted for. Furthermore, due to the long study period, differences in treatment over time and across regions could have affected these results. Nevertheless, the association between treatment and prognosis remained significant after adjustments for PS status, TNM stage, age, sex, TS, and occupational disease. None of the patients received multimodality treatment, but different lines of treatments were given when the cancer progressed. Interestingly, a subset of LTS patients (9%) did not receive any kind of therapy, primarily due to their own choice. The fact that some patients had prolonged survival in the absence of any therapy underlies the heterogeneity and indolent behavior of some of the tumors. Thus, prospective, preferably randomized, studies are needed to fully study the role of therapies in this heterogenous disease.

6.2 Computed tomography assessment and survival

Because of its wide availability and limited cost, CT is the primary imaging modality to assess the extension of MPM and the follow-up after treatment. In study I, we evaluated MPM patients' CT characteristics at the time of diagnosis. In line with previous studies, we found that the most common CT findings were pleural thickening among with pleural effusion and pleural plaques or calcification [41]. In accordance with previous findings, most patients were diagnosed at a late stage [106].

Mesothelioma evaluation by CT is challenging due to its' unique morphology, which is characterized by uniform or separate lesions that extend from the pleural surface to the surrounding tissues with variable thickness. This leads to the well-known limitations regarding tumor burden assessment and staging using current imaging modalities. TNM stage T-category refers to the invasion into adjacent structures rather than the actual tumor size. Moreover, assessing T-category from CT-images is subjective and CT tends to underestimate the true tumor extent, which leads to discrepancies when compared to pathological stage [107]. Also, many kinds of incidental findings (such as lung nodules, atelectasis, and adrenal incidentalomas) are relative common in CT. These incidental findings cannot easily be classified as metastases (M1) or benign findings (M0) by CT alone. Thus, current guidelines suggest using multiple imaging modalities and possible invasive biopsies in the staging process [8,9]. Limitations in the CT assessment and relatively small sample sizes may explain why TNM stage did not show prognostic utility after adjustments in our study. Indeed, a larger study consisting of 2141 MPM patients from IASLC database found that both pathological stage and clinical stage were independent prognostic factors in different multivariate models [106]. However, they also reported discrepancies between pathological and clinical staging, which have launched a search for other quantitative methods in order to improve clinical staging.

Multiple approaches, such as tumor volumetry or tumor thickness measurements have been proposed to replace or add to the T-category of the TNM stage [5,6]. From these, tumor volumetry is a more

comprehensive and better validated method than measuring tumor thickness. In addition, one study combined tumor volumetry and maximal tumor thickness in the interlobar fissures and found that this combination outperformed clinical TNM staging in prognostic evaluation [322]. However, quantifying exact tumor volume from CT images can be complicated, time-consuming, and several different image softwares have been used in previous studies [89]. In addition, distinguishing mesothelioma from the adjacent structures on CT can be a subjective process and prone to errors even if computer assistance is used [91]. In study I, we proposed a novel and practical tumor burden (TS) estimation, which accounts for maximal tumor thickness as well as tumor pleural extension. This idea arose from a previously published method used for evaluating pleural abnormalities such as pleural plaques [323]. After our publication, a study evaluated the prognostic value of tumor volumetry, TNM stage, and number of macroscopic tumor sites (i.e. pleural extent), and found that they all predicted survival by similar effect and that the combination of tumor volume and pleural extent was the most powerful prognostic factor [239]. The advantages of our method are that it is practical and easy to measure and has excellent intra-observer agreement. The disadvantage is that it is only a crude estimate of the actual size. Despite this, we observed an association with sarcomatoid histology and TNM T- and N-classes. In survival analyses, it was an independent predictor for survival in study I as well as in another cohort in study IV.

Other CT-characteristics that associated with survival in study I were the thickness of pleural effusion, tumor laterality, and the presence of pleural calcification. From these, the amount of pleural effusion remained significant after adjustments. Even if previous studies have found similar associations, the reasons for this remains unclear [41]. We hypothesize that the presence of effusion might reflect the biological activity of the disease. However, measuring the thickness is a crude estimation of the real amount of effusion and prone to errors such as previous thoracentesis.

The current standard for MPM response evaluation is mesothelioma-specific mRECIST criteria, which combines six perpendicular unidimensional tumor thickness measurements from three separate sections of the pleural tumor [90]. This criterion has been criticized for its high interobserver variability and imprecision in measurements. Thus, a revision was made in 2018 with several refinements and clarifications on the methodology [91]. Also, alternative approaches such as measuring changes in linear tumor thickness or tumor volume have been used as a marker for treatment response [324–326]. In study II, we used TS change for treatment response evaluation and found a very strong correlation when compared to the original mRECIST criteria.

6.3 Circulating activins and follistatins in MPM

In study II, we investigated the levels of circulating activin A and B as well as their endogenous antagonists` follistatin and FSTL3 in patients with thoracic malignancy. We observed that particularly levels of activin A were elevated in MPM patients compared to NSCLC and benign lung lesions. Similar findings have been found in previous preclinical studies and in one clinical study [10,12]. In the latter, Hoda et al reported activin A levels to be an independent prognostic factor in MPM patients associated with histological subtypes and CT-based tumor volume. Our sample size was not large enough for a prognostic evaluation and we did not find any differences between the histological groups. However, we observed several clinically meaningful associations, which could be seen as a surrogate for survival.

An international expert panel defined cancer cachexia as a multifactorial syndrome with ongoing loss of skeletal muscle mass, with or without loss of fat mass, that cannot be reversed by nutritional support and leads to progressive functional impairment [167]. It is associated with various physiological and metabolic changes, such as systemic inflammation, leading to reduced tolerance to

cancer therapies and poor outcome. Indeed, it is considered to be responsible for up to 20% of all cancer deaths [167]. Previous studies have reported widely varying estimates for the prevalence of cancer cachexia in other thoracic cancers such as NSCLC, while little data is available in MPM patients [312]. We observed that over half of MPM patients were defined as cachectic at diagnosis. This is not surprising, since the presence of weight loss, a hallmark in cachexia, is a common condition in MPM with negative prognostic impact [212]. In comparison to MPM, we found that only 12% NSCLC patients were cachectic at diagnosis. This difference might be explained by the different disease stages in MPM and lung cancer patients.

Out of these biomarkers, activin A had the most clinical value. In line with previous preclinical studies and clinical studies in colorectal and lung cancer patients, activin A was the only marker associated with cancer cachexia [13,14]. The association remained significant after adjustments on inflammation marker CRP, age, and sex. The link between cachexia and systemic inflammation has been firmly established, even though the exact mechanisms are unclear. Interestingly, the results of anti-inflammatory medication have been variable in reversing cachexia, whereas inhibition of activin receptor signaling or blocking activin via soluble follistatin have withdrawn cachexia changes in animal as well as clinical studies without affecting inflammatory markers [14,172,178].

In addition to the relation between activin A and cancer cachexia, we observed that activin A levels were positively correlated with pretreatment TS. This is in line with previous work, where a similar correlation was observed between activin A and tumor volumetry [12]. In the exploratory analysis, they also observed that patients who had a dramatic decrease in plasma activin A levels after chemotherapy experienced also a major therapeutic response. We also looked at chemotherapy response after a report where activin A was identified as a potential mediator for platinum-based chemotherapy resistance [15]. In that study, it was observed that blocking activin signaling overcame chemoresistance as well as prevented cisplatin-induced nephrotoxity in lung adenocarcinoma models. Our exploratory findings are comparable to these associations, since the patients with progressive disease after chemotherapy had almost three times higher activin A levels compared to patients with partial response or stable disease. This finding remained significant after adjustments, but it needs to be validated in larger cohorts.

6.4 Histopathological factors and overall survival

Histological subtypes are one of the strongest prognostic factors in MPM. Epithelioid is the most frequent histopathological subtype with the best prognosis [25]. In study III, we analyzed the histopathological features predictive of prolonged survival in patients within the epithelioid subgroup. We used previously published characters and sought to identify novel prognostic features.

Before analyzing prognostic features, we made a thorough assessment of the original MPM diagnosis. These steps included a clinical, radiological and histopathological evaluation in order to confirm the initial diagnosis. We performed additional IHC stains, including *BAP1*, which is considered to be helpful in distinguishing benign mesothelial proliferation from malignant [17]. However, the loss of *BAP1* is observed approximately in 60% of epithelioid mesotheliomas and it is also a common finding in other malignancies [327]. To our surprise, more LTS tumors where *BAP1* negative than controls, although the difference was not significant. Since patients with germline *BAP1* mutations have better prognosis and exhibit similar clinical features than we observed in our LTS group (low asbestos burden, young age, more female), it is possible that some of the LTS patients have an inherited *BAP1* germline mutation [83,328]. However, other common features in hereditary cancer syndromes, such as a family history of cancer, other *BAP1*-related cancers, or prior mesotheliomas in the family were

absent in most of the cases. Indeed, some authors have proposed that if some of these features are present, genetic testing for *BAP1* should be considered, leading to possible mesothelioma screening or routine monitoring within the family [328]. Unfortunately, the germline mutations from this cohort could not be assessed due to the lack of adequate samples.

In our cohort, the proportion of patients exceeding five-year survival is equivalent to previous population based studies (approximately 5%) [211]. In contrast to our original hypothesis, we found that the accuracy of the original diagnosis was good in the LTS cohort. Only one malignancy was found to be a non-mesothelioma, which was the result of miscoding at the cancer registry. We could also confirm two LMMs and two WDPMs that were originally diagnosed as diffuse MPM. One explanation for the good diagnostic accuracy is that some of the cases were originally evaluated in the nationwide mesothelioma expert panel. In addition, at least in the university hospitals, all of the new mesothelioma cases are evaluated in a multidisciplinary team in order to solve diagnostic and treatment issues. However, it could be concluded that the main reason for diagnostic accuracy appears to be the familiarity for this diagnosis among the pathologists.

LMMs are histologically indistinguishable from MPM but don't share similar diffuse pleural spread. Compared to MPM, LMMs can be potentially extirpated with R0 resection, leading to favorable prognosis [17,203]. Thus, even though the differential diagnosis can be challenging, it is crucial to separate diffuse MPM from its localized counterpart. As discussed by Allen et al., sometimes early presentation of MPM as a single dominant mass can mimic LMMs, whereupon only careful observation can distinguish these diagnoses [202].

The differential diagnosis of WDPM and diffuse MPM is challenging and somewhat unsettled. Histological distinction is based on cytological and/or architectural atypia with stromal invasion: true WDPMs have usually no, or only limited, invasion in contrast to MPM, where tumor invasion is a central part of the diagnosis [17]. Recently, *BAP1* negativity has been suggested to be incompatible with WDPM [108]. In addition to two WDPMs with no invasion, the diagnosis of WDPM with minimal invasive foci could be considered in three of our cases [205]. However, these cases showed clinical behavior compatible with MPM (*BAP1* loss when available, MPM as a cause of death, metastasis, or additional biopsy with profound invasion), and thus were included in the study as diffuse MPMs. WDPMs are rare variants of mesothelioma, which are typically found in the peritoneum in young women [329]. It is defined as a tumor with papillary architecture and bland cytology with superficial spread [17]. WDPMs typically have an indolent clinical behavior, especially if located in the peritoneum, while some have reported that pleural WDPMs can progress to diffuse MPMs [330]. We identified a WDPM-like polypoid pattern in part of the MPM patients, called an exophytic polypoid growth, which was almost only seen in LTS tumors and proved to be an independent prognostic factor in multivariate analysis. In addition, we noted that WDPMs with no or limited invasion exhibited a similar outcome than the MPMs with polypoid growth pattern. However, due to small numbers in each category, further studies are required on this aspect.

The three-tier nuclear grade was another independent prognostic factor in multivariate analyses. In the LTS group nuclear grade I was present in over twice that of the control group, whereas grade II was most common observation in controls. Several studies both in pleural and peritoneum epithelioid mesothelioma have obtained similar results [245,331]. In addition, a multi-institutional study revealed that OS could be further stratified when nuclear grade was added to the presence of necrosis [246]. This was also noted in a recent multidisciplinary expert proposal for pathologic update, where it was suggested that all epithelioid tumors should be graded either into low-grade (nuclear grade I and II without necrosis) or high-grade (nuclear grade II with necrosis or nuclear grade III) based on the combination of nuclear grade and necrosis [108]. In contrast to nuclear grade, we found that the

presence of necrosis had prognostic utility only in univariate analyses. Since the proportion of necrosis found in these tumors was low, we could not reliably test the combination with nuclear grade. We also observed an association between fiber concentration found in lung tissue and nuclear grade. This was driven by the positive correlation with mitotic count. Mitosis is a process of cell duplication in which cell divides and produces identical copies of itself [332]. Thus, an increased mitotic count can be seen as a universal characteristic of cancerous growth, whereas the evaluation of nuclear atypia is a more subjective measure of nucleus size and shape.

Other histopathological features that were more common in LTS and associated with survival in univariate analyses but not after adjustments were morphological subtypes, the presence of single layer, and low tumor density in the tissue sample. Out of these features, morphological subtypes have been mostly studied in mesothelioma. Heterogeneity in the epithelioid subtype and its association with survival was shown by Kadota et al [185]. They observed that a pleomorphic subtype had the worst survival (OS 8.1 months) followed by solid (OS 13.7 months), micropapillary (OS 15.8 months), tubulopapillary (OS 17.9 months), and trabecular (OS 24.9 months) subtypes. They proposed that the first three could be combined as high-grade subtypes and trabecular and tubulopapillary as low-grade. We used these combinations for survival analyses due to the small number of tumors in some subgroups. The caveat of this method is that mesotheliomas are heterogenous and these subtypes are classified as a predominant growth, whereas some tumors can exhibit several different growth patterns. However, since they are reproducible, easy to perform, and have a clear survival association, we think that they should be included into routine histopathological evaluation. The growth of mesothelium in a single layer in tubular structures and the proportion of malignant mesothelial cells to other tissues within the diagnostic sample are novel features that have not been previously reported. We think that the first is related to the degree of differentiation in the tumor and analogous to well-differentiated adenocarcinomas, whereas the latter is an approximation of the density of the malignant cells in the diagnostic sample. They can both be affected by the size and site of tumor biopsy and thus challenging to import into clinical practice.

6.5 Strengths and limitations

The data collected in studies I and III-IV were based on a compilation of different registries including patient information from hospital medical records. In addition, a thorough evaluation of CT images and histopathological samples was performed. We think that using multiple ways to collect data makes the results more comprehensive. Nevertheless, the major limitation in these studies is the retrospective nature of a major part of the study design. This increases the potential bias in data collection, such as missing or incorrect information, and prevents us from drawing definite causality inferences [333]. However, several attempts were made in order to improve the data quality. For example, the radiological and histopathological features were assessed blinded to the patient information, several confounding factors were controlled, and the follow-up for patients was almost complete.

Another major limitation of these studies is the small study population size, which is due to the rarity of the disease. Even if we used a nationwide approach in studies III-IV, we lacked statistical power, especially in the subgroup analyses. Thus, different stage groups, ECOG PS-groups, and epithelioid histopathological subtypes needed to be merged for the survival analyses. This weakens the generalizability of these results. In addition, the low amount of MPM patients in study II prevented us from studying the prognostic significance of the circulating biomarkers.

Even if the CT scans used in our studies were performed in the same hospital district, multiple different protocols and techniques were used, which increases variability in the analyses. In study I,

only a single radiologist evaluated the CT images, thus inter-reader agreement was not measured. Having two independent radiologists would allow us to be more confident in the reproducibility of the CT characteristics used in these studies. However, we assessed intra-rater agreement by reviewing a subgroup of the images in a random order.

Similarly, in study III and IV, one pathologist was in charge of scoring the histological samples, and a second opinion was asked in cases of uncertain findings. Since some of the features are subjective, an external validation is needed in order to properly verify these findings. In contrast to some studies that had used primarily larger surgical specimen [245], we mainly evaluated the first available biopsy. This approach can be seen as a strength as these findings are better applicable in the clinical setting, where samples are far from standardized. However, small or bad quality specimens can complicate the accurate evaluation and may influence the histopathological classification of a heterogeneous disease such as MPM.

We investigated how different therapies associated with survival in study IV. The role of therapy cannot be reliably determined in non-randomized studies, due to numerous confounding factors that cannot be adjusted for [333]. In addition, there can also be substantial regional differences in the therapies offered to MPM patients and some treatment options have evolved in the 10-year study period.

The fact that our LTS patients had lower TS and were lower staged at the diagnosis than the control group rises the possibility of lead-time bias. This usually occurs in screening studies, where a diagnostic approach identifies the disease earlier and creates an impression of prolonged survival without significant modification of the disease course [334]. This effect is difficult to measure or adjust for in retrospective studies. However, we think that it does not significantly contribute to the findings of our studies, given the dismal prognosis in MPM in general and the fact that no screening was used.

Finally, some selection bias may have occurred in forming the control group in studies III-IV. This was based on the practical approach using an already characterized cohort of patients from HUS region with an epithelioid histology. In contrast, the LTS group was formed from the nationwide cancer registry. Regional differences in the diagnosis and treatment might have affected patient outcome. Similarly, the assignment of presurgical patients in study II leaded to a disproportion in different stage groups in NSCLC and MPM. However, we do not think that this significantly modified the results, since we did not find a clear correlation between the biomarkers and TNM stage.

7. Conclusions

The combination of tumor thickness and pleural extension was an independent prognostic factor in MPM. Besides mortality, it associates with histological subgroups and TNM stage.

Circulating activins and follistatins levels were elevated in MPM compared to NSCLC and benign lung tumors. Out of these markers, activin A was the best one at separating these different diagnostic groups. We found that cancer cachexia is a prevalent condition in MPM at diagnosis. In MPM patients, activin A levels correlated with tumor size, cancer cachexia, and poor chemotherapy response.

We identified 52 patients with epithelioid histology with survival of over five years. Contrary to our expectations, we did not find a single case where another type of malignancy would have been misdiagnosed as a mesothelioma. However, one patient was miscoded at the cancer registry and four cases were found to represent other types of mesothelial tumors than diffuse MPM. Independent predictors for prolonged survival were ECOG performance status, tumor size, polypoid growth pattern, and nuclear grade.

8. Implications and future perspectives

Results obtained in this doctoral study may aid to determine the prognosis of MPM. Whether these factors affect the feasibility of various treatment modalities remains to be studied.

Our studies bring forward some challenges related to CT evaluation of current T-category in the TNM-staging. Using quantitative measurements, such as tumor thickness, volumetry, or TS-estimation, as proposed in our studies, could better stratify patients into different stage groups.

The role of activins, particularly activin A, needs to be further studied in MPM. Several confounding factors make it unpractical for diagnostic purposes, but the association that we found in our studies could open a novel pathway for treatment that could lead to palliation of symptoms and possible therapeutic effects.

We found that asbestos exposure does not only predispose to MPM but also associates with survival. However, in these studies its prognostic utility vanished after adjustments, which might be because of the small sample size. Thus, we plan to further study this association in a large-scale study.

More precise histological diagnosis improves risk stratification and could help in patient selection for certain treatments in the future. Due to the rarity and heterogeneity of the MPM tumor, some of the histopathological prognostic features could be difficult to implement in the clinical use. Therefore, we aim to test whether artificial intelligence (AI) could be used to analyze scanned tumor samples and if it could predict survival from the histological slides used in studies III and IV. Furthermore, we aim to investigate the tumor microenvironment and the genetic landscape of these tumors to further search for prognostic associations.

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